ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 329 (2000) 539–547

CARBOHYDRATE RESEARCH

The synthesis, testing and use of 5-fluoro-α-D-galactosyl fluoride to trap an intermediate on green coffee bean α-galactosidase and identify the catalytic nucleophile

Hoa D. Ly [a], Steven Howard [a], Kelly Shum [a], Shouming He [a], Alex Zhu [b], Stephen G. Withers [a,*]

[a] *Department of Chemistry, University of British Columbia, Vancouver, BC, Canada V6T 1Z1*
[b] *The Lindsley F. Kimball Research Institute of The New York Blood Center, New York, NY 10021, USA*

Received 14 June 2000; accepted 11 July 2000

Abstract

5-Fluoro-α-D-galactopyranosyl fluoride was synthesized and its interaction with the active site of an α-galactosidase from green coffee bean (*Coffea arabica*), a retaining glycosidase, characterized kinetically and structurally. The compound behaves as an apparently tight binding ($K_i = 600$ nM) competitive inhibitor, achieving this high affinity through reaction as a slow substrate that accumulates a high steady-state concentration of the glycosyl-enzyme intermediate, as evidenced by ESiMS. Proteolysis of the trapped enzyme coupled with HPLC/MS analysis allowed the localization of a labeled peptide that was subsequently sequenced. Comparison of this sequence information to that of other members of the same glycosidase family revealed the active site nucleophile to be Asp145 within the sequence LKY**D**NCNNN. The importance of this residue to catalysis has been confirmed by mutagenesis studies.

Keywords: 5-Fluoro-α-D-galactopyranosyl fluoride; Slow substrate; α-Galactosidase; Covalent glycosyl-enzyme intermediate; Mass spectrometry

1. Introduction

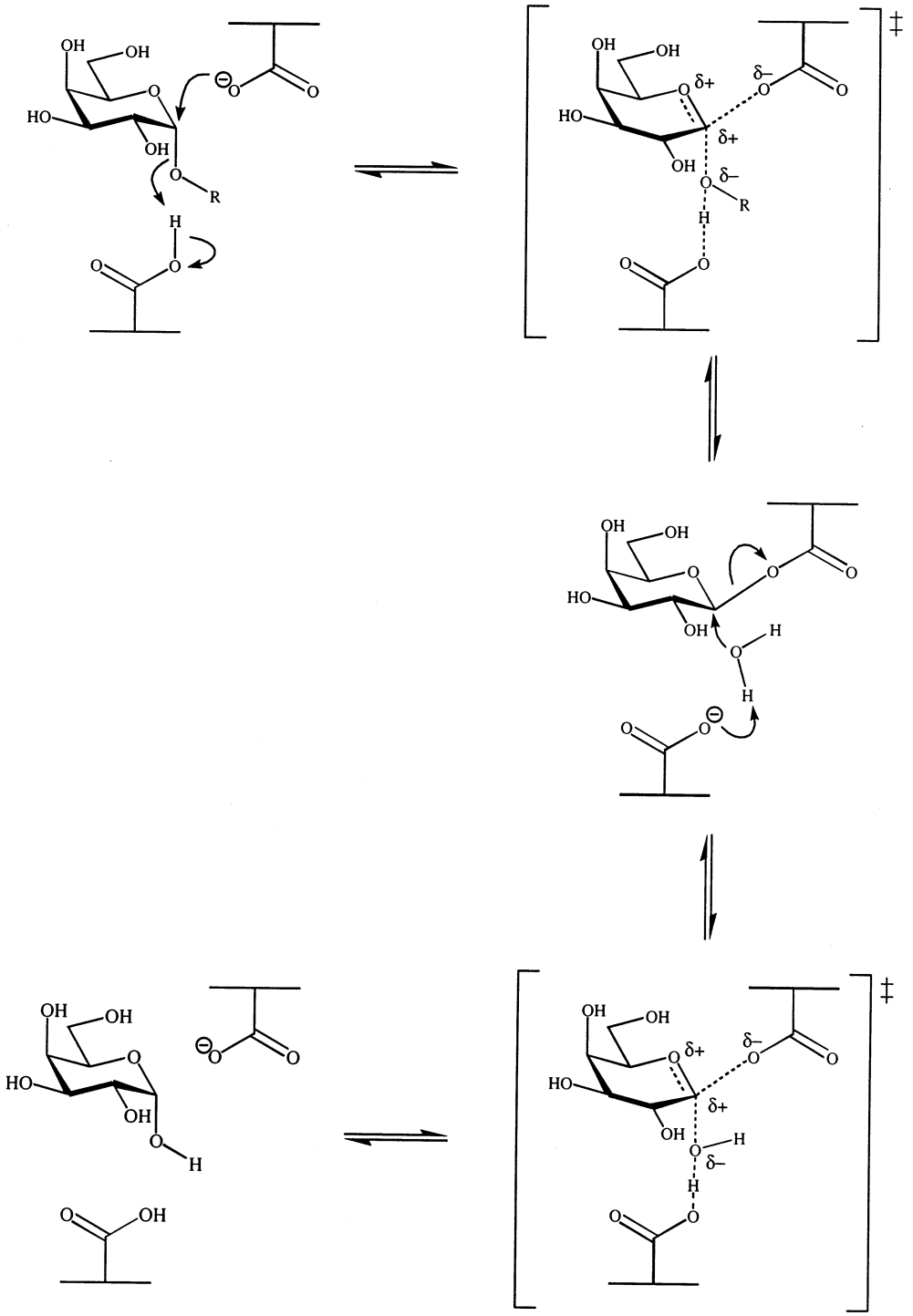
α-Galactosidases (EC 3.2.1.22) from a variety of sources [1–4] are exoglycosidases capable of cleaving α-linked galactose residues from glycoconjugates. The enzyme isolated from green coffee beans has been well characterized and is shown to be capable of hydrolyzing a broad spectrum of substrates [5] to yield products of retained anomeric configuration [6]. The broad substrate specificity of this enzyme has proved to be especially useful in the in vitro conversion of group B red blood cells to group O red blood cells given that the only structural difference between the two antigens is an additional terminal α-linked galactose residue on antigen B [7,8]. To meet the demands for the amount of enzyme required for this process, the full-length cDNA encoding green coffee bean α-galactosidase has been isolated [9] and high level expression of the protein has been achieved [10].

Based on the stereochemical outcome of the reaction, green coffee bean α-galactosidase is classified as a retaining glycosidase and is therefore believed to follow a double displacement mechanism whereby a covalent glycosyl-enzyme intermediate is formed and

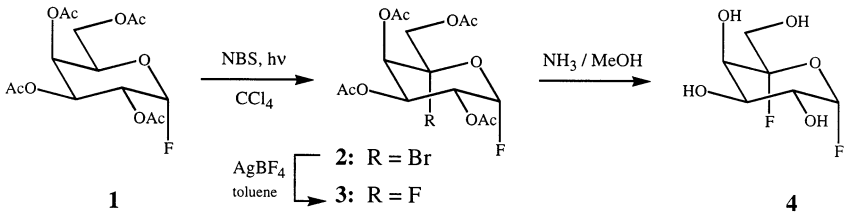
* Corresponding author. Tel.: +1-604-8223402; fax: +1-604-8222847.
E-mail address: withers@chem.ubc.ca (S.G. Withers).

0008-6215/00/$ - see front matter
PII: S0008-6215(00)00214-7

hydrolyzed via oxacarbenium ion-like transition states (Scheme 1). In the study of retaining glycosidases, fluorosugars have proved to be invaluable tools. In particular, activated 2-deoxy-2-fluoroglycosides have played pivotal roles in the inactivation of a number of β-glycosidases and the subsequent identification of their catalytic nucleophiles [11–14]. These mechanism-based inactivators function by forming relatively stable glycosyl-enzyme intermediates. The presence of a highly electron withdrawing fluorine substituent inductively destabilizes the oxacarbenium ion-like transition states through which both the gly-



Scheme 1.



Scheme 2.

cosylation and deglycosylation steps proceed. As a consequence, both steps of the double displacement mechanism are slowed. However, through incorporation of a good leaving group at C-1, the glycosylation rate can be increased, allowing the 2-deoxy-2-fluoroglycosyl-enzyme intermediate to be trapped. While these 2-fluoro-substituted inactivators have worked well for trapping the covalent intermediate of retaining β-glycosidases, their use with retaining α-glycosidases has not met with the same success; rather, they act as slow substrates for which the glycosylation step is rate-limiting [15,16].

Recently, a new class of mechanism-based inhibitors, the 5-fluoroglycosyl fluorides, has been synthesized and shown to inactivate both α- and β-retaining glycosidases [17]. Since that time, the covalent glycosyl-enzyme intermediates of two retaining α-glycosidases, an α-glucosidase from yeast and an α-mannosidase from jack bean have been trapped using compounds of this class and the catalytic nucleophiles of each enzyme identified [18,19]. Based on sequence alignments, glycosidases have been grouped into a number of different families [20]. To date, the active site nucleophiles of 21 glycosidases representing 13 different families of glycosyl hydrolases have been identified with the aid of both the 2-deoxy-2-fluoro and 5-fluorosugar inactivators [21]. Of these families, 11 are β-retaining enzymes and only two (the Family 13 α-glucosidase and the Family 38 α-mannosidase) are α-retaining glycosidases. Here, we report the synthesis and kinetic evaluation of 5-fluoro-α-D-galactosyl fluoride as a potential mechanism-based inactivator for use in the trapping of the covalent glycosyl-enzyme intermediate of the Family 27 green coffee bean α-galactosidase. We also report the preliminary identification of the catalytic nucleophile through proteolysis, LC/MS analysis and through comparisons with other enzymes of this family.

2. Results and discussion

Synthesis.—The synthesis of 5-fluoro-α-D-galactopyranosyl fluoride **4** from 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl fluoride **1** [22], which was readily obtained from the per-O-acetylation of galactose [23] followed by treatment with HF/pyridine [24], is outlined in Scheme 2. A key step in the sequence was the free radical photobromination of **1** to yield **2**. This reaction is known to be highly regio- and stereoselective, with reaction occurring primarily at the tertiary carbon of the 5-position of per-O-acetylated glycopyranosyl fluorides [25–27] to yield the kinetically and thermodynamically favoured product where the bromine is in the axial orientation [27]. Fluorination of the resulting 5-bromogalactosyl fluoride **2** followed by deacetylation afforded the desired compound **4**.

Initial attempts to fluorinate **2** using silver fluoride were unsuccessful, despite previously reported successes with the corresponding D-*gluco* and D-*manno*-epimers [17,19]. This fluorination was eventually accomplished using silver tetrafluoroborate in toluene yielding **3** as the product of retained configuration at C-5 with no detectable formation of the 5-fluoro-L-*altro* epimer, which would be the product of inverted configuration at C-5. The assignment of stereochemistry at C-5 is based on the assumption that the favoured conformation of **3** is a $^4C_1$ chair while that of the 5-fluoro-L-*altro* epimer is most likely a boat (Fig. 1). In each case, the conformation is favoured by the anomeric effect of the fluorine substituent at C-5 and it also allows the bulky

6-*O*-acetyl group of each epimer to adopt a sterically less demanding equatorial position. In the boat conformation, H-4 and F-5 of the 5-fluoro-L-*altro* epimer should have a large coupling constant of 20–30 Hz due to their transdiaxial relationship. Since no H-4, F-5 coupling was observed in the product of the fluorination reaction, it was concluded that the sole epimer formed in this case was that which had the D-*galacto* configuration at C-5. Support for this conclusion was later provided by the results of performing the fluorination in diethyl ether. Under these conditions, a mixture of the D-*galacto* and L-*altro* epimers in an approximately 1:1 ratio (^{1}H and ^{19}F NMR) were obtained. Unfortunately, these two products could not be separated but from the NMR data, one set of peaks was identical to those of **3**. In the second set of peaks, a large coupling constant of 29.7 Hz was indeed observed for H-4, thereby validating the assumption that the favoured conformation of the L-*altro* epimer is a boat.

Two explanations are possible for the observed retention of configuration when **2** is fluorinated in toluene, which differs from that seen for sugars with an equatorial acetate substituent at C-4 when mixtures of the two epimers are typically obtained [28]. One possibility is that neighbouring group participation by the axial 4-*O*-acetyl group of **2** yields a 4,5-acetoxonium ion intermediate that can only undergo attack at C-5 from the bottom face, yielding the D-*galacto* epimer. Alternatively, it may be that **3** is simply the thermodynamically favoured product from the fluorination of **2** and that equilibration of the epimers occurs in this case. The boron trifluoride present in the reaction mixture could certainly act as the Lewis acid catalyst for this epimerization reaction. As mentioned earlier, when diethyl ether was used as the solvent, in place of toluene, both the D-*galacto* and L-*altro* products were indeed obtained. Presumably, when ether is used as the solvent, all of the boron trifluoride is complexed by the excess ether, thereby decreasing its capacity to function as a Lewis acid catalyst. In contrast, no complexation of boron trifluoride occurs in toluene. As a result, this Lewis acid is free to catalyze the aforementioned epimerization reaction to yield the most stable product. However, it is not clear why equilibration would occur more rapidly in the *galacto* than the *gluco* series, thus participation of a neighbouring group seems to be at least partially responsible for the outcome.

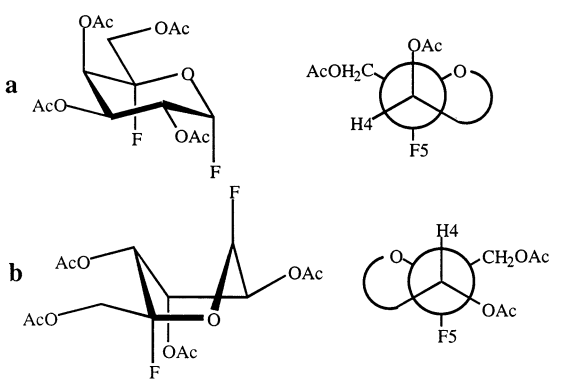
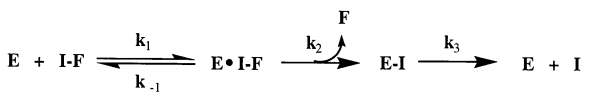


Fig. 1. Conformations of the (a) 5-fluoro-D-*galacto* epimer and the (b) 5-fluoro-L-*altro* epimer. Haworth projections are shown along the C-4, C-5 axis.

Enzymology.—The reaction of **4** with green coffee bean α-galactosidase is best described by the kinetic model shown in Scheme 3 where E represents free enzyme, I-F represents 5-fluoro-α-D-galactopyranosyl fluoride, E-I is the glycosyl-enzyme intermediate and the products of the reaction are represented by I and F. According to this model, an accumulation of the intermediate E-I should occur, when the rate constant for the formation of this intermediate (k_2) is significantly higher than that for its breakdown (k_3). As a result of the intermediate accumulating, inactivation of the enzyme in a time-dependent manner may occur if k_3 is slow relative to the time required for assay.



Scheme 3.

When **4** was tested as an inactivator of green coffee bean α-galactosidase, no time-dependent inactivation of the enzyme was observed. Instead, the enzyme was found to be reversibly inhibited by the compound in a competitive manner (Fig. 2), yielding an apparent K_i value of 0.6 μM when assayed against the substrate, *p*-nitrophenyl α-D-galactopyranoside (PNP-Gal, $K_m = 0.45$ mM). This

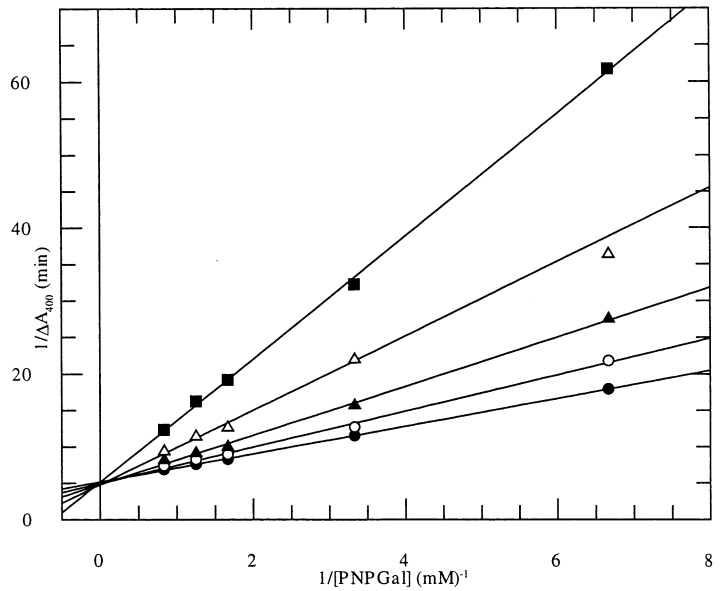


Fig. 2. Double reciprocal plot showing the inhibition of green coffee bean α-galactosidase by 5-fluoro-α-D-galactopyranosyl fluoride at the following concentrations: 0.19 (●), 0.38 (○), 0.75 (▲), 1.50 (△) and 3.00 μM (■).

apparent 750-fold tighter binding of **4**, in comparison with PNP-Gal, by the enzyme is unusual given that PNP-Gal contains an aromatic aglycone, which typically binds tightly to glycosidase active sites, while **4** does not. As such, the apparent tight binding of **4** observed is most likely not a result of enhanced non-covalent binding interactions, but is rather a kinetic phenomenon resulting from an accumulation of the intermediate ($k_2 > k_3$). The absence of any observed time-dependent inactivation therefore suggests that although turnover of the intermediate (k_3) is slow relative to its formation (k_2), the deglycosylation step is still much faster than the time scale of the assay, thus turnover occurs during the early stage of aliquot removal and kinetic analysis.

Evidence in support of an accumulated intermediate was provided by electrospray mass spectral studies (ESiMS) on α-galactosidase incubated in the presence of **4**. Shown in Fig. 3(a) is the mass spectrum of green coffee bean α-galactosidase, the two peaks at 39,622 and 39,771 Da representing the two different isoforms of the enzyme. When the enzyme was incubated with **4**, a mass increase in both of the isoforms was observed (Fig. 3(b)). The mass difference between each set of native and inhibited enzymes was found to be 180 ± 2 Da, a value which is consistent with the addition of a single 5-fluorogalactosyl label (181 Da). This direct trapping of the glycosyl-enzyme intermediate is not only demonstrative of its persistent presence at steady state, but also confirms that this intermediate is covalent in nature.

To identify the catalytic nucleophile involved in the formation of the covalent fluoroglycosyl-enzyme intermediate, the labeled enzyme was subjected to proteolysis by pepsin and the resulting peptide containing the label was purified and sequenced. Because of the instability of the 5-fluorogalactosyl label, the amount of time allotted for proteolysis was limited to 15 min, since prolonged digestion times resulted in loss of the label. As an initial control, a sample of protein treated with 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-*lyxo*-hexopyranoside, a known inactivator of α-galactosidases [29], was similarly digested. The resulting peptides from each digest were separated by LC/ESiMS and localization of both the 5-fluorogalactosyl and the 2-deoxy-2,2-difluoro-*lyxo*-hexopyranosyl peptides was accomplished through comparative mapping with respect to a peptic digest of unlabeled protein. Thus, comparison of LC/ESiMS profiles revealed that a 6710 Da fragment present in the digest of unlabeled enzyme was largely absent in the labeled samples. In its place, peptides of mass 6890 and 6893 Da

corresponding to the respective 5-fluorogalactosyl and 2-deoxy-2,2-difluoro-*lyxo*-hexopyranosyl labeled samples were found. Unfortunately, the large size of these peptides did not permit them to be sequenced by tandem mass spectrometry and all attempts to further proteolyze these peptides were unsuccessful or resulted in loss of the label. Therefore, the corresponding unlabeled peptide was fully purified by HPLC/ESiMS and subjected to N-terminal amino acid sequencing by Edman degradation, yielding the sequence FASWGVDYL. From this result, the 6710 Da peptide was assigned the sequence 134-FASWGVDYLKYDNCNNNNISPKERYPIMSKALLNSGRSIFFSLCEWGEEDPATWAKEV-191 to match the observed mass. Of the eight carboxylic acid residues present in this peptide, only Asp140 and Asp145 are conserved amongst the glycosidases of Family 27 (Fig. 4) making them the most likely candidates to act as the catalytic nucleophile. Recently, the catalytic nucleophile from another member of this family, the *Phanerochaete chrysosporium* α-galactosidase was unequivocally identified as Asp130 in the mature form of this protein (Asp150 of the preprotein) within the sequence YLKYDNC [30]. Based on the sequence alignment shown in Fig. 4, Asp145 of green coffee bean α-galactosidase is therefore assigned the role of the catalytic nucleophile. Indeed, mutation of Asp145 to an asparagine residue yielded a completely inactive enzyme (A. Zhu, unpublished data), demonstrating the importance of this residue to catalysis.

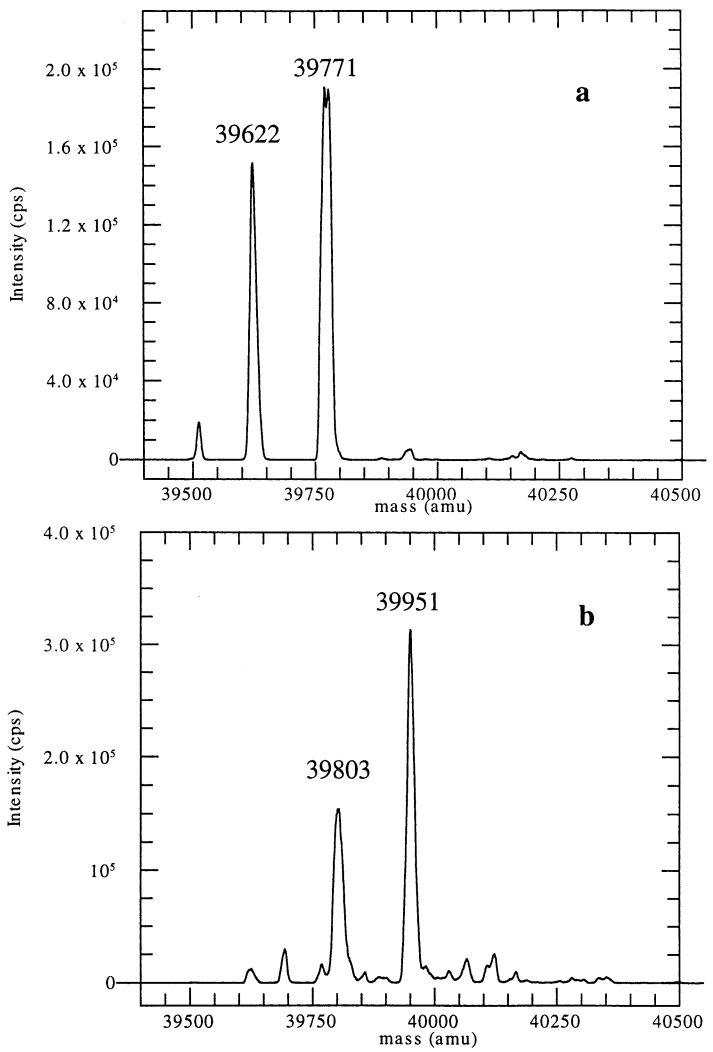


Fig. 3. Electrospray mass spectrum of green coffee bean α-galactosidase (a) in the absence and (b) presence of 5-fluoro-α-D-galactopyranosyl fluoride.

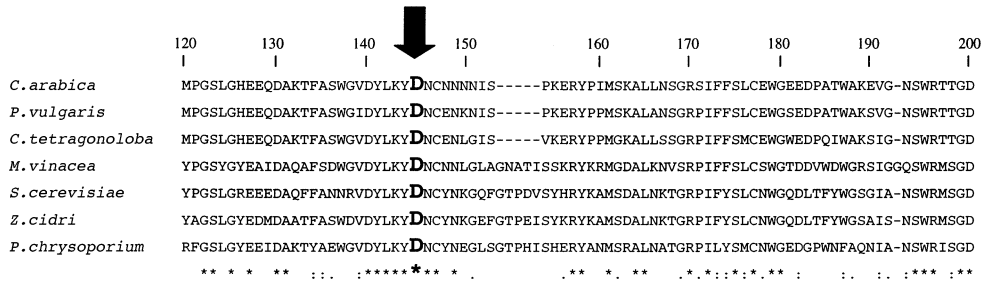


Fig. 4. A partial sequence alignment of various representative α-galactosidases from Family 27 using ClustalX. The sources of these protein sequences and their entries in the Swiss-Prot Database are as follows: *Coffea arabica* (green coffee bean, Q42656), *Phaseolus vulgaris* (Q41100), *Cyamopsis tetragonoloba* (P14749), *Mortierella vinacea* (Q02402), *Saccharomyces cerevisiae* (P41947), *Zygosaccharomyces cidri* (Q99172) and *Phanerochaete chrysoporium* [29]. Numbering of the amino acids is based on that of *C. arabica*.

3. Conclusions

We have reported the synthesis of 5-fluoro-α-D-galactosyl fluoride and shown it to behave as an apparent tight binding competitive inhibitor of green coffee bean α-galactosidase. This apparent tight binding ($K_i = 600$ nM) is consistent with this compound reacting as a slow substrate causing an accumulation of the glycosyl-enzyme intermediate, as was also evidenced by ESiMS. Fortunately, the lifetime of this 5-fluorogalactosyl-enzyme intermediate was sufficiently long to allow for proteolysis and then isolation of the labeled peptide. A partial N-terminal amino acid sequence of this peptide was subsequently obtained, and through sequence alignments, Asp145 was identified as the catalytic nucleophile of green coffee bean α-galactosidase.

4. Experimental

General methods.—All buffer chemicals and other reagents were obtained from Sigma/Aldrich Chemical Co. unless otherwise noted. All ^{1}H NMR spectra were recorded at either 200 or 400 MHz using a Brüker AC-200 or WH-400 spectrometer (chemical shifts are quoted relative to $CHCl_3$ or CH_3OH) and all ^{19}F NMR spectra were recorded at 188 MHz using the Brüker AC-200 spectrometer with CF_3CO_2H as the reference. High resolution desorption chemical ionization mass spectrometry (HRMS-DCI) was performed on a Delsi-Nermag R10-10C mass spectrometer using ammonia as the reagent gas. Thin layer chromatography was performed on E. Merck precoated aluminium-backed sheets of Silica Gel 60 F_{254} with detection being effected by heating with 10% ammonium molybdate in 2 M H_2SO_4. Column chromatography was performed under positive pressure using Silica Gel 60 (230–400 mesh) from BDH Inc.

2,3,4,6-Tetra-O-*acetyl-5-bromo*-α-D-*galacto-pyranosyl fluoride* (**2**).—A suspension of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl fluoride (**1**) [22] (2.0 g, 5.7 mmol) and *N*-bromosuccinimide (4.1 g, 22.8 mmol) in anhyd CCl_4 (50 mL) was heated to reflux under nitrogen by means of two 200-W household light bulbs. After 9 h, the reaction was allowed to cool and then filtered. The filtrate was washed with aq $NaHCO_3$ (2×50 mL) and water (2×50 mL), dried ($MgSO_4$) and concentrated under diminished pressure. The residue was chromatographed over silica gel (69:1 CH_2Cl_2–EtOAc) to yield **2** (1.28 g, 52%) as a colourless gum. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 6.03 (dd, 1 H, $J_{3,2}$ 11.2, $J_{3,4}$ 2.9 Hz, H-3), 5.95 (dd, 1 H, $J_{1,F}$ 53.4, $J_{1,2}$ 3.3 Hz, H-1), 5.78 (d, 1 H, $J_{4,3}$ 2.9 Hz, H-4), 5.26 (ddd, 1 H, $J_{2,F}$ 23.4, $J_{2,3}$ 11.2, $J_{2,1}$ 3.3 Hz, H-2), 4.52 (d, 1 H, $J_{6a,6b}$ 11.9 Hz, H-6a), 4.32 (d, 1 H, $J_{6b,6a}$ 11.9 Hz, H-6b), 2.00-2.12 (s, 12 H, $4 \times$ OAc). ^{19}F NMR ($CDCl_3$, 188 MHz): δ −70.5 (dd, $J_{F,1}$ 53.4, $J_{F,2}$ 23.4 Hz). HRMS-DCI: Calc. for $C_{14}H_{18}O_9F^{79}Br + NH_4^+$: 446.04619, Found: 446.04645; Calc. for $C_{14}H_{18}O_9F^{81}Br + NH_4^+$: 448.04415, Found: 448.04343.

2,3,4,6-Tetra-O-acetyl-5-fluoro-α-D-galactopyranosyl fluoride (**3**).—To a solution of **2** (0.25 g, 0.58 mmol) in anhyd toluene (5 mL) and 4 Å sieves was added silver tetrafluoroborate (0.27 g, 1.37 mmol) and the reaction was stirred under an atmosphere of nitrogen. After 40 min, the reaction was filtered through Celite and rinsed with EtOAc. The filtrate was then washed with aq $NaHCO_3$ (25 mL) and brine (25 mL), dried ($MgSO_4$) and the solvent evaporated under diminished pressure. Purification by silica gel chromatography (CH_2Cl_2) yielded **3** (29 mg, 14%) as a colourless gum. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 5.85 (dd, 1 H, $J_{1,F\text{-}1}$ 53.1, $J_{1,2}$ 3.0 Hz, H-1), 5.60–5.67 (m, 2 H, H-3, H-4), 5.23 (ddd, 1 H, $J_{2,F\text{-}1}$ 23.1, $J_{2,3}$ 10.8, $J_{2,1}$ 3.0 Hz, H-2), 4.42 (dd, 1 H, $J_{6a,F\text{-}5}$ 25.5, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.06 (dd, 1 H, $J_{6b,F\text{-}5}$ 12.3, $J_{6b,6a}$ 12.3 Hz, H-6b), 2.00–2.11 (s, 12 H, 4 × OAc). ^{19}F NMR ($CDCl_3$, 188 MHz): δ −38.1 (ddd, $J_{F\text{-}5,6a}$ 25.5, $J_{F\text{-}5,F\text{-}1}$ 23.9, $J_{F\text{-}5,6b}$ 12.3 Hz, F-5), −64.9 (ddd, $J_{F\text{-}1,1}$ 53.1, $J_{F\text{-}1,2}$ 23.1, $J_{F\text{-}1,F\text{-}5}$ 23.9 Hz, F-1). HRMS-DCI: Calc. for $C_{14}H_{18}O_9F_2 + NH_4^+$: 386.12627, Found: 386.12690.

5-Fluoro-α-D-galactopyranosyl fluoride (**4**).—A solution of **3** (46 mg, 0.13 mmol) in anhyd MeOH under an atmosphere of nitrogen was cooled to 0 °C. Ammonia gas was bubbled into the reaction for 5 min, after which the reaction vessel was sealed and allowed to warm to r.t. After 2.5 h, the reaction was shown to be complete by TLC. The solvent was subsequently evaporated under diminished pressure and the residue was chromatographed over silica gel (49:1 EtOAc–MeOH) to yield **4** (14 mg, 57%) as a colourless syrup. ^{1}H NMR (CD_3OD, 400 MHz): δ 5.58 (dd, 1 H, $J_{1,F\text{-}1}$ 54.4, $J_{1,2}$ 3.2 Hz, H-1), 4.13 (dd, 1 H, $J_{4,F\text{-}5}$ 2.7, $J_{4,3}$ 1.9 Hz, H-4), 4.02 (ddd, 1 H, $J_{3,2}$ 10.5, $J_{3,F\text{-}5}$ 2.4, $J_{3,4}$ 1.9 Hz, H-3), 3.93 (ddd, 1 H, $J_{2,F\text{-}1}$ 25.0, $J_{2,3}$ 10.5, $J_{2,1}$ 3.2 Hz, H-2), 3.79 (dd, 1 H, $J_{6a,F\text{-}5}$ 23.3, $J_{6a,6b}$ 12.2 Hz, H-6a), 3.59 (dd, 1 H, $J_{6b,F\text{-}5}$ 14.2, $J_{6b,6a}$ 12.2 Hz, H-6b). ^{19}F NMR ($CDCl_3$, 188 MHz): δ −44.82 (ddd, $J_{F\text{-}5,6a}$ 23.3, $J_{F\text{-}5,F\text{-}1}$ 22.5, $J_{F\text{-}5,6b}$ 14.2 Hz, F-5), −66.98 (ddd, $J_{F\text{-}1,1}$ 54.4, $J_{F\text{-}1,2}$ 25.0, $J_{F\text{-}1,F\text{-}5}$ 22.5 Hz, F-1). HRMS-DCI: Calc. for $C_6H_{10}O_5F_2 + NH_4^+$: 200.08400, Found: 200.08346.

Enzyme kinetics.—The expression and purification of green coffee bean α-galactosidase have been reported previously [31]. All kinetic studies were performed at 37 °C in 50 mM sodium phosphate buffer, pH 6.5 containing 0.1% bovine serum albumin. Reactions were initiated by the addition of enzyme (final concentration = 0.3 μg/mL), bringing the total volume to 200 μL. Reaction rates were measured by monitoring the release of *p*-nitrophenolate ($\lambda = 400$ nm, $\varepsilon = 7.28 \times 10^{-3}$ M^{-1} cm^{-1}) from PNP-Gal by means of a UNICAM 8700 UV–Vis spectrophotometer equipped with a circulating water bath. Inhibition studies were performed by varying concentrations of both PNP-Gal and **4**. While the data are represented in this paper in the form of a double reciprocal plot, the apparent K_i value was actually calculated by direct fit of the initial rates to the equation below using Grafit version 3.0 [32].

$$v = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]}$$

Labelling and LC/ESMS analysis of protein.—Green coffee bean α-galactosidase (2.7 mg/mL) was incubated either in the absence or presence of 5-fluoro-α-D-galactopyranosyl fluoride (2.7 mM) for 1 min in 50 mM sodium phosphate, pH 6.5 to give a final volume of 15 μL. The sample was then injected onto an Ultrafast Microprotein Analyser (Michrom BioResources, Pleasanton, CA, USA) equipped with a PLRP-S 1 × 50 mm reverse phase column. The protein was subsequently eluted with a 2–90% gradient of MeCN in water and 0.05% trifluoroacetic acid at a flow rate of 50 μL/min over 5 min. Mass analysis of the eluted protein was performed using a PE-Sciex API 300 triple-quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada).

Proteolysis and LC/ESMS analysis of peptides.—To a sample of green coffee bean α-galactosidase (2.7 mg/mL), either unlabeled or labeled according to the above procedure, was added a solution of pepsin (1:10 w/w) in 150 mM sodium phosphate, pH 2.0, doubling the volume of the reaction to 30 μL. This mixture was allowed to stand at r.t. for 15 min before

it was quickly frozen at −78 °C while awaiting further manipulations. Once thawed, the samples were quickly injected onto an Ultrafast Microprotein Analyser (Michrom BioResources) equipped with a 3.9 × 150 mm Waters Delta Pak C18 column. Separation of the peptides was achieved by elution with a 0–60% gradient of MeCN in water and 0.05% trifluoroacetic acid at a flow rate of 500 μL/min over 60 min. Through the use of a post-column flow splitter, 90% of the sample was diverted into a fraction collector and the remainder was sent for analysis in a PE-Sciex API 300 triple-quadrupole mass spectrometer (Sciex). The quadrupole mass analyser was scanned over a mass-to-charge ratio range of 300–2400 amu, with a step size of 0.5 amu and a dwell time of 1 ms per step. The ion source potential was set at 5 kV; the orifice energy was 50 V.

Peptide sequencing by Edman degradation.—N-Terminal amino acid sequencing of peptides isolated by LC/ESiMS according to the conditions described above was performed by S. C. Perry of the Nucleic Acids/Protein Services (NAPS) Unit at the University of British Columbia (Vancouver, Canada). After the samples were lyophilized and resuspended in 0.1% trifluoroacetic acid, they were adsorbed onto a 0.45 μm PDF membrane and fixed with Biobrene. Sequencing was then carried out on an Applied Biosystems 476A Sequencer and the degradation products (phenylthiohydantoin–amino acid derivatives) were identified by comparison of retention times to standards eluted from a PE 120A HPLC equipped with a 2.1 × 220 μm C18 PTH column.

Acknowledgements

The authors would like to thank Dr H. Brumer III and Dr M.L. Sinnott for their generous gift of 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-*lyxo*-hexopyranoside. Financial support from the Natural Sciences and Engineering Research Council of Canada is also gratefully acknowledged. A. Zhu was supported in part by grant HL55482-01A1 from the NIH.

References

[1] P.M. Dey, E.M. Del Campill, R.P. Lazica, *J. Biol. Chem.*, 258 (1983) 923–929.
[2] K.J. Dean, C.C. Sweeley, *J. Biol. Chem.*, 254 (1979) 9994–10000.
[3] Y. Uda, S.C. Li, Y.T. Li, *J. Biol. Chem.*, 252 (1977) 5194–5200.
[4] J. Hata, M. Dhar, M. Mitra, M. Harmata, F. Haibach, P. Sun, D. Smith, *Biochem. Int.*, 28 (1992) 77–86.
[5] F. Yagi, A.E. Eckhardt, I.J. Goldstein, *Arch. Biochem. Biophys.*, 280 (1990) 61–67.
[6] W. Weiser, J. Lehmann, H. Matsui, C.F. Brewer, E.J. Hehre, *Arch. Biochem. Biophys.*, 292 (1992) 493–498.
[7] N. Harpaz, H.M. Flowers, N. Sharon, *Arch. Biochem. Biophys.*, 170 (1975) 676–683.
[8] I.J. Goldstein, G. Siviglia, R. Hurst, L. Lenny, *Science*, 215 (1982) 168–170.
[9] A. Zhu, I.J. Goldstein, *Gene*, 140 (1994) 227–231.
[10] A. Zhu, C. Monahan, Z.K. Wang, *Biochim. Biophys. Acta*, 1297 (1996) 99–104.
[11] S.G. Withers, R.A.J. Warren, I.P. Street, K. Rupitz, J.B. Kempton, R. Aebersold, *J. Am. Chem. Soc.*, 112 (1990) 5887–5889.
[12] D. Tull, S.G. Withers, N.R. Gilkes, D.G. Kilburn, R.A.J. Warren, R. Aebersold, *J. Biol. Chem.*, 266 (1991) 15621–15625.
[13] J.C. Gebler, R. Aebersold, S.G. Withers, *J. Biol. Chem.*, 267 (1992) 11126–11130.
[14] L.F. Mackenzie, G.S. Brooke, J.F. Cutfield, P.A. Sullivan, S.G. Withers, *J. Biol. Chem.*, 272 (1997) 3161–3167.
[15] S.G. Withers, K. Rupitz, I.P. Street, *J. Biol. Chem.*, 263 (1988) 7929.
[16] J. McCarter, M. Adam, C. Braun, M. Namchuk, D. Tull, S.G. Withers, *Carbohydr. Res.*, 249 (1993) 77.
[17] J.D. McCarter, S.G. Withers, *J. Am. Chem. Soc.*, 118 (1996) 241–242.
[18] J.D. McCarter, S.G. Withers, *J. Biol. Chem.*, 271 (1996) 6889–6894.
[19] S. Howard, S. He, S.G. Withers, *J. Biol. Chem.*, 273 (1998) 2067–2072.
[20] B. Henrissat, *Biochem. J.*, 280 (1991) 309–316.
[21] D.L. Zechel, S.G. Withers, in C.D. Poulter (Ed.), *Comprehensive Natural Products Chemistry*, Elsevier, New York, 1999, pp. 279–314.
[22] W.A. Szarek, G. Grynkiewicz, B. Doboszewski, G.W. Hay, *Chem. Lett.*, (1984) 1751–1754.
[23] M.L. Wolfrom, A. Thompson, *Methods Carbohydr. Chem.*, 2 (1963) 212.
[24] M. Hayashi, S.-i. Hashimoto, R. Noyori, *Chem. Lett.*, (1984) 1747–1750.
[25] R.J. Ferrier, P. Tyler, *J. Chem. Soc., Perkin Trans. 1*, (1980) 1528.
[26] J.P. Praly, G. Descotes, *Tetrahedron Lett.*, 28 (1987) 1405.
[27] L. Somsak, R.J. Ferrier, *Adv. Carbohydr. Chem. Biochem.*, 49 (1991) 37.
[28] A.W.P.C. Wong, personal communication.
[29] H. Brumer III, P.F.G. Sims, M.L. Sinnott, *Biochem. J.*, 339 (1999) 43–53.
[30] D.O. Hart, S. He, C.J. Chany II, S.G. Withers, P.F.G. Sims, M.L. Sinnott, H. Brumer III, *Biochemistry*, 39 (2000) 9826–9836.
[31] A. Zhu, C. Monahan, Z. Zhang, R. Hurst, L. Leng, I.J. Goldstein, *Arch. Biochem. Biophys.*, 324 (1995) 65–70.
[32] R.J. Leatherbarrow, Grafit Version 3.0, 1990, Erithacus Software Ltd., Staines, UK.