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Note

N-Bromoacetyl-glycopyranosylamines as affinity labels for a β-glucosidase and a cellulase

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There has been an enormous increase in interest in glycosidases in recent years, fuelled largely by the biotechnological potential of such enzymes as cellulases and amylases in the degradation of biomass. As a consequence, a very large number of glycosidases have been cloned and sequenced, allowing their assignment into families based upon sequence alignments[1]. Unfortunately, as yet, very little structural information is available on these enzymes from X-ray crystallographic analysis. Structures solved to date are those of polysaccharide degrading enzymes such as lysozymes[2], amylases[3], and cellulases[4,5]; no structures for glycosidases which hydrolyse monosaccharide glycosides have yet been solved.

An alternative approach to the identification of important active-site residues in glycosidases involves the use of mechanism-based inactivators or of affinity labels. Examples of mechanism-based inactivators of glycosidases include the conduritol epoxides[6], the glycosylmethyl triazenes[7], the 2-deoxy-2-fluoro-glycosides[8,9], and to some extent, the dihaloalkyl glycosides[10] which function by release of an aglycon which is a potent electrophile. Examples of affinity labels include glycosyl epoxide derivatives[6], glycosyl isothiocyanates[11], and *N*-bromoacetyl-glycosylamines[12–15]. Two examples of this latter category of affinity labels have been synthesized and tested; *N*-bromoacetyl-β-D-galactosylamine as an inactivator of *E. coli* β-galactosidase[12,13,15] and *N*-bromoacetyl-β-D-glucosylamine as an inactivator of *Aspergillus wentii* β-glucosidase[14], though this latter work was only reported in a preliminary fashion. In this manuscript, we describe the synthesis and detailed characterisation of both *N*-bromoacetyl-β-D-glucosylamine and *N*-bromoacetyl-β-cellobiosylamine, and the evaluation of their efficacy as affinity labels for a β-glucosidase and a cellulase, respectively.

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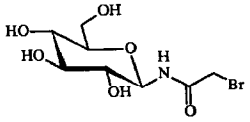
EXPERIMENTAL

General methods and materials.—Melting points were determined on a Laboratory Devices Mel-temp II melting-point apparatus and are uncorrected. Solvents and reagents used were either reagent grade, certified, or spectral grade. Reactions were monitored by thin layer chromatography using Merck Kieselgel 60 F-254 analytical plates. Compounds were visualized by charring with 10% H_2SO_4 in MeOH. Flash chromatography was performed using columns of Kieselgel 60 (180–230 mesh). ^1H NMR spectra were recorded on 200 MHz Bruker AC-200 or 400 MHz WH-400 spectrometers. Chemical shifts (δ) were recorded for solutions in D_2O and CD_3OD and were measured against external 4,4-dimethyl-4-silapentane-1-sulphonate (DSS). Cellobiose, D-glucose and buffers were obtained from Sigma Chemical Company. Synthesis of benzyl 4-*O*-(β-D-glucopyranosyl)-1-thio-β-D-xylopyranoside (BGTX) will be described elsewhere. Purifications of *Agrobacterium* β-glucosidase[17] and *Cellulomonas fimi* exoglucanase[19] were carried out as described previously.

N-*Bromoacetyl-β-D-glucopyranosylamine*(**1**).—Bromoacetic anhydride[15] (450 mg, 1.74 mmol) was added to a solution of β-D-glucopyranosylamine[18] (150 mg, 0.84 mmol) in DMF (0.9 mL) over 5 min. The mixture was stirred for 30 min at room temperature, poured over ice-cold anhyd ether (45 mL), then stirred for 1 h. The ether was decanted and the remaining gum crystallized from MeOH to yield **1** as a white solid (30 mg, 12%); mp 165–167°C, $[\alpha]_D^{20} + 4.7°$(*c* 0.5, H_2O); ^1H NMR (D_2O): δ 3.95 (s, 2 H, CH_2BrCO), 3.84 (dd, 1 H, $J_{6,6'}$ 12, $J_{6,5}$ 2 Hz, H-6), 3.69 (dd, 1 H, $J_{6',6}$ 12, $J_{6',5}$ 5 Hz, H-6′), 3.39 (t, 1 H, $J_{3,2}$ 9, $J_{3,4}$ 9 Hz, H-3), 3.25 (t, 1 H, $J_{2,1}$ 9, $J_{2,3}$ 9 Hz, H-2), 3.28–3.35 (m, 2 H, H-4, H-5). Anal. Calcd for $C_8H_{15}NO_6Br$: C, 32.02; H, 4.70; N, 4.67. Found: C, 31.96; H, 4.88; N, 4.75.

N-*Bromoacetyl-β-cellobiosylamine* (**2**).—Cellobiose (1.0 g, 2.79 mmol) was dissolved in water (10 mL), ammonium bicarbonate was added until the solution was saturated, and the solution stirred for 10 days with periodic resaturation with



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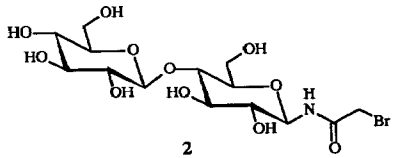

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ammonium bicarbonate. Evaporation of the solvent under reduced pressure yielded a white gum. Partial purification by flash chromatography (2:2:1 EtOAc–MeOH–H_2O) gave a mixture of β-cellobiosylamine and cellobiose. Bromoacetic anhydride (900 mg, 3.46 mmol) was then added to a solution of this mixture in DMF (10 mL) and stirred at room temperature for 3 h. The solution was poured over ice-cold anhyd. ether and stirred for 1 h. Purification by flash chromatography (5:2:1 EtOAc–MeOH–H_2O) and crystallization from MeOH–diethyl ether gave **2** as a white solid (582 mg, 45%); mp 143–145°C, $[\alpha]_D^{20}$ – 1.6 (*c* 0.7, H_2O); ^1H NMR (CD_3OD): δ 4.80 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.41 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 3.87 (s, 2 H, CH_2BrCO), 3.25–3.90 (m, 11 H, H-2,3,4,5,6^a,6^b,3',4',5',6'^{a},6'^{b}), 3.22 (t, 1 H, $J_{2',1'}$ 8, $J_{2',3'}$ 8 Hz, H-2'). Anal. Calcd for $C_{14}H_{24}NO_{11}Br$: C, 36.36; H, 5.19; N, 3.03. Found: C, 36.35; H, 5.40; N, 2.95.

Enzyme inactivation study.—Inactivation parameters for *C. fimi* exoglucanase with **2** were determined by incubating the enzyme in sodium phosphate buffer (50 mM, pH 7) containing bovine serum albumin (1 mg/mL) and varying concentrations (0.8, 1.6, 3.2, 4.0, 4.8 mM) of the inactivator at 37°C. Aliquots of the inactivation mixture were removed at different time intervals, diluted into reaction cells containing a large volume of substrate (2,4-dinitrophenyl β-cellobioside, 7 mM = $10 \times K_m$), and residual enzyme activity determined by monitoring the rate of release of dinitrophenolate anion. Inactivation was monitored until 90% of the enzyme activity was lost in each case. Slopes of plots of the natural logarithm of residual enzyme activity versus time yielded pseudo-first order rate constants for inactivation at each inactivator concentration. Values for K_i (inactivator dissociation constant) and k_i (inactivation rate constant) were calculated from plots of the reciprocal of the pseudo-first-order rate constants versus the reciprocal of the inactivator concentration. These data were fitted using the programme GraFit[16].

Protection against exoglucanase inactivation by **2** was studied as follows. A sample of exoglucanase was incubated in sodium phosphate buffer containing BSA (1 mg/mL), **2** (3.5 mM), and the competitive inhibitor BGTX (0 or 9.7 mM). Aliquots were removed at different time intervals, diluted into reaction cells containing saturating concentrations of substrate, and the residual enzyme activity monitored as described above. Pseudo-first order rate constants for inactivation at each BGTX concentration were calculated and compared to determine the degree of protection afforded by BGTX against inactivation by **2**.

Inactivation kinetic parameters for *Agrobacterium* β-glucosidase using **1** were determined similarly. Samples of β-glucosidase were incubated in sodium phosphate buffer containing BSA and **1** (0.8, 2.2, 6.3, 11.1, 19.00, and 27.0 mM) at 37°C. Aliquots were removed and residual enzyme activity was monitored using *p*-nitrophenyl β-D-glucopyranoside as substrate at saturating concentrations (1 mM = $12 \times K_m$). Protection experiments involved incubation of solutions containing the β-glucosidase and **1** (19 mM) in the presence and absence of β-glucopyranosyl benzene (24.3 mM). Inactivation parameters were calculated as previously described.

Stoichiometry of incorporation of inactivator.—The stoichiometry of inactivation for β-glucosidase with **1** and that for the exoglucanase with **2** were determined as follows. A sample of the enzyme (~ 100 μg) was incubated with the inactivator at 37°C until it was 70–90% inhibited. Aliquots of labelled and unlabelled enzymes (~ 10 μg) were injected into a PE SCIEX API III Ion Spray LC/MS system and mass spectra were recorded. From the mass difference between the labelled and unlabelled enzyme samples, the stoichiometry of incorporation of the inactivator was determined.

RESULTS AND DISCUSSION

Both the exoglucanase and the β-glucosidase were inactivated by their corresponding *N*-bromoacetyl-glycopyranosylamines according to pseudo-first-order kinetics as shown in Figs. 1 and 2 and the kinetic parameters determined are shown in Table I. Protection against inactivation by competitive inhibitors was shown in both case (Figs. 1c and 2c). Addition of 9.7 mM BGTX (K_i = 3.0 mM) to a sample of exoglucanase containing *N*-bromoacetyl-β-cellobiosylamine (3.5 mM) reduced the observed inactivation rate from 6×10^{-2} to 1.2×10^{-2} min^{-1}. Likewise, addition of 24.5 mM glucosyl benzene (K_i = 3.4 mM)[17] to a sample of β-glucosidase containing *N*-bromoacetyl-glucosylamine (19 mM) reduced the observed inactivation rate from 4×10^{-3} to 2×10^{-3} min^{-1}. In both cases the rate reduc-

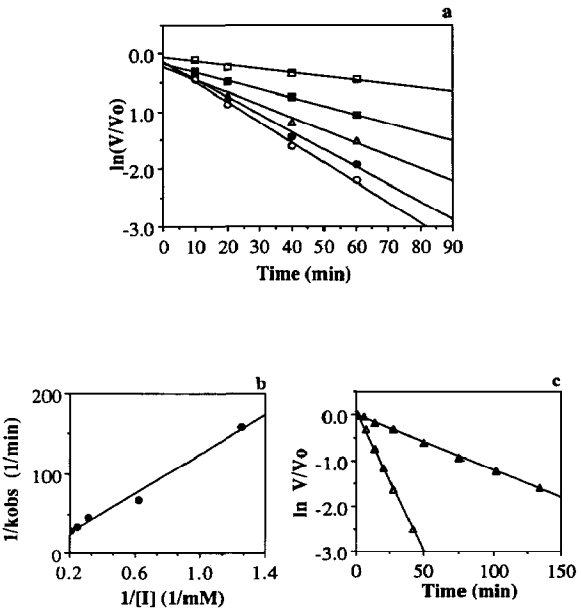

Fig. 1. Inactivation of *C. fimi* exoglucanase by *N*-bromoacetyl-β-cellobiosylamine (**2**). (a) Semi-logarithmic plot of residual activity versus time at the following inactivator concentrations: (□), 0.8 mM; (■), 1.6 mM; (△), 3.2 mM; (●) 4.0 mM; (○), 4.8 mM. (b) Double-reciprocal plot of pseudo-first-order rate constants from (a). (c) Protection against inactivation given by 0 mM (△) and 9.7 mM (▲) BGTX.

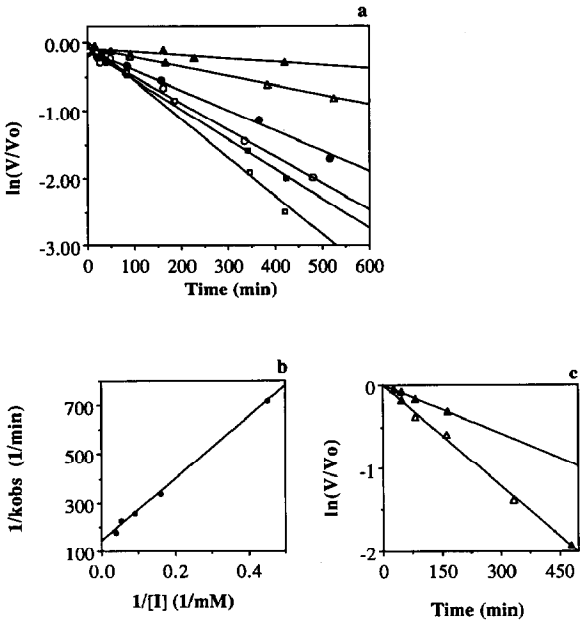

Fig. 2. Inactivation of *Agrobacterium sp.* β-glucosidase by *N*-bromoacetyl-β-D-glucopyranosylamine (**1**). (a) Semi-logarithmic plot of residual activity versus time at the following inactivator concentrations: (▲), 0.8 mM; (△), 2.2 mM; (●), 6.3 mM; (○), 11.1 mM; (■), 19.0 mM; (□), 27.0 mM. (b) Double-reciprocal plot of pseudo-first-order rate constants from (a). (c) Protection against inactivation given by 0 mM (△) and 24.5 mM (▲) β-D-glucopyranosylbenzene.

tions observed are essentially as expected if the competitive inhibitor and the inactivator compete for the same site. These results suggest that the inactivation process results in blocking of the active site in each case and therefore likely involves residues present at the active site of each enzyme; thus these compounds may prove useful for the identification of catalytically important residues.

However, since these derivatives are relatively reactive affinity labels rather than the more specific mechanism-based inactivators, it is possible that more than one site on the enzyme could be derivatized, even though the inactivation kinetics would suggest a simple first-order process. This would be quite possible if labelling of other sites had no effect on the activity of the enzyme, but it would result in

TABLE I

Inactivation kinetic parameters

Enzyme	Inactivator	K_i (mM)	k_i (min^{-1})	k_i/K_i ($min^{-1}\ mM^{-1}$)
β-Glucosidase	*N*-BrAc-GluNH [a] (**1**)	8.3	0.0072	8.7×10^{-4}
Exoglucanase	*N*-BrAc-CbNH [b] (**2**)	9.1	0.083	9.1×10^{-3}

[a] *N*-BrAc-GluNH, *N*-bromoacetyl-β-D-glucopyranosylamine. [b] *N*-BrAc-CbNH, *N*-bromoacetyl-β-cellobiosylamine.

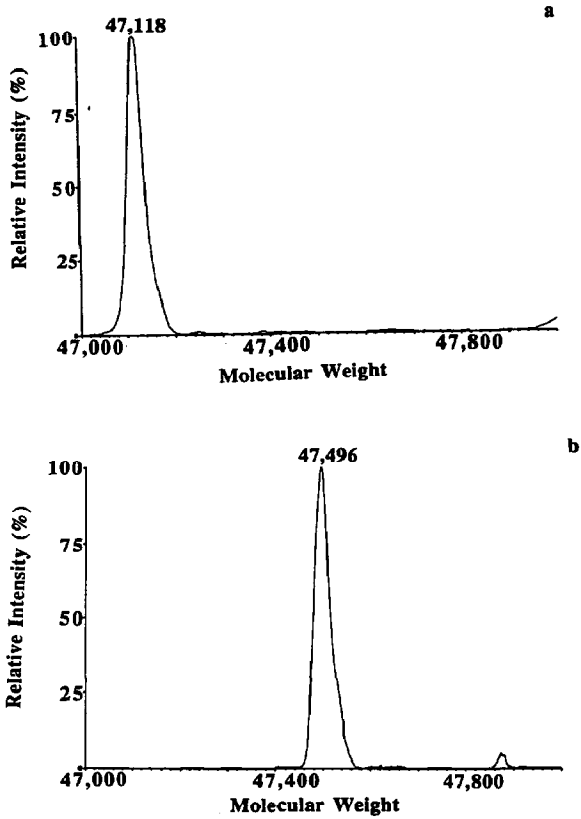

Fig. 3. Ion spray mass spectrometry of *C. fimi* exoglucanase. (a) Reconstructed mass spectrum of unlabelled exoglucanase; (b) reconstructed mass spectrum of *N*-bromoacetyl-β-cellobiosylamine-inactivated exoglucanase.

considerable ambiguity in subsequent sequencing studies. In order to investigate this possibility, the stoichiometry of inactivation in each case was determined by means of ion spray mass spectrometry of the inactivated enzyme sample. As can be seen in Fig. 3, the molecular weight of the exoglucanase prior to inactivation was $47\,118 \pm 7$ while that of the inactivated enzyme was $47\,496 \pm 5$. The increase in molecular weight of 378 corresponds well, within experimental error, to the mass increase of 382 expected upon derivatisation with a single *N*-acetyl-cellobiosylamine moiety. The very small peak observed at 47 873 corresponds to an enzyme species to which two inactivator moieties have become attached; this could be suppressed by using shorter incubation times. Complete inactivation of the exoglucanase can therefore be achieved upon reaction with a single equivalent of inactivator. By contrast, inactivation of the β-glucosidase by *N*-bromoacetyl-glucosylamine results in the incorporation of multiple inactivators as seen in the ion spray mass spectrum shown in Fig. 4. The mass spectrum of the β-glucosidase is further complicated by the presence of two native β-glucosidase species of masses 51 205 and 51 066, corresponding to species with and without an *N*-terminal

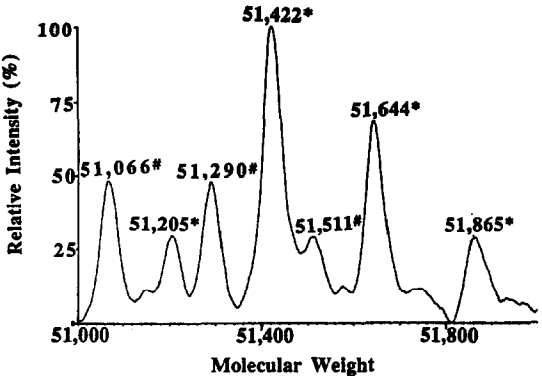

Fig. 4. Ion spray mass spectrometry of *Agrobacterium* β-glucosidase. (a) Reconstructed mass spectrum of unlabelled and *N*-bromoacetyl-β-D-glucopyranosylamine-inactivated β-glucosidase: (*) corresponds to (+)Met β-glucosidase and (#) to (−)Met β-glucosidase.

methionine residue. The presence of this *N*-terminal methionine residue, due to incomplete processing at the higher expression levels employed, has previously been shown to have no effect on kinetic parameters. Inactivation of the (+)Met β-glucosidase by *N*-bromoacetyl-glucosylamine results in protein species of masses 51 422, 51 644, and 51 865, corresponding to the incorporation of one, two, and three *N*-acetyl-glucosylaminyl moieties, respectively, of molecular weight 220. Interestingly, it would appear that only one and two *N*-acetyl-glucosylaminyl moieties are incorporated into the (−)Met β-glucosidase, possibly indicating that one of the residues being labelled in the (+)Met β-glucosidase is the *N*-terminal methionine. This is particularly attractive in light of the fact that the residue labelled in β-galactosidase using this strategy was also a methionine. Attempts to inactivate the enzyme more selectively using lower concentrations of *N*-bromoacetyl-glucosylamine for longer times were unsuccessful, multiple incorporations being observed in each case.

It therefore seems unlikely that *N*-bromoacetyl-glucosylamine can be used successfully to identify active site residues in the β-glucosidase. However, such experiments with the exoglucanase using *N*-bromoacetyl-cellobiosylamine are quite feasible and should allow the identification of a residue which may be important to catalysis or substrate binding, through sequencing of labelled proteolytically derived peptides. This will provide useful insights into structure–function relationships in this enzyme, particularly in conjunction with an investigation of the properties of mutants of this enzyme modified at the labelled amino acid. This inactivator may well also prove useful for similar studies on other cellulases.

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