Serratia marcescens chitobiase is a retaining glycosidase utilizing substrate acetamido group participation

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The stereochemistry of the reaction catalysed by *Serratia marcescens* chitobiase was determined by HPLC separation of the anomers of *N*-acetylglucosamine produced during the hydrolysis of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (PNP-GlcNAc). In the early stages of the reaction, the β-anomer was found to prevail, whereas the α-anomer dominated at mutarotation equilibrium. This established that chitobiase hydrolyses glycosidic bonds with overall retention of the anomeric configuration. Chitobiase-catalysed hydrolysis of PNP-GlcNAc was competitively inhibited by a series of chito-oligosaccharides (degree of polymerization 2–5) that were selectively de-*N*-acetylated at their non-reducing end. The results are in accord with the participation of the acetamido group at C-2 of the substrate in the catalytic mechanism of chitobiase and related enzymes.

INTRODUCTION

Chitobiase (*N*-acetyl-β-D-glucosaminide *N*-acetylglucosaminohydrolase, EC 3.2.1.30) is the enzyme that hydrolyses terminal, non-reducing GlcNAc residues in chitobiose and higher chito-oligosaccharides. The *Serratia marcescens* enzyme belongs to glycoside hydrolase family 20, which also comprises human *N*-acetylhexosaminidase, the heritable deficiency of which is responsible for Tay–Sachs disease [1]. The three-dimensional structure of *S. marcescens* chitobiase in complex with chitobiose has been solved [2] and features a distorted skew-boat conformation for the GlcNAc residue in the catalytic subsite. The unusual conformation of the bound sugar and the lack of a suitably located enzyme nucleophile suggested that chitobiase utilizes a molecular mechanism involving the participation of the acetamido group of GlcNAc [2], as had once been proposed for hen egg-white lysozyme (HEWL) [3–5]. A similar indication of the possible participation of the acetamido group of the substrate in a retaining plant chitinase was inferred from the three-dimensional structure analysis of allosamidin (Figure 1) bound to hevamine [6]. Whereas chitotriose binds to hevamine at subsites -4 to -2, the oxazoline-like residue at the 'reducing' end of the pseudo-trisaccharide allosamidin was found in the -1 subsite mimicking the catalytic intermediate formed with a participating acetamido group. Sulzenbacher et al. [7] have recently shown that the carbonyl oxygen of the GlcNAc residue at the cleavage site of chitobiase lies in a position corresponding exactly to that found for the enzymic nucleophile of a cellulase. This observation provided strong additional evidence for the neighbouring participation (anchimeric assistance) of the acetamido group of the substrate in the catalytic reaction mechanism of chitobiase. The recent report that GlcNAc-thiazoline (Figure 1) acts as an excellent competitive inhibitor of jack bean *N*-acetyl-β-hexosaminidase shows that acetamido participation also prevails in the mechanism of this related enzyme [8]. If a retaining GlcNAc-cleaving enzyme uses the acetamido group for anchimeric assistance in the cleavage of the β-glycosidic bond, then *N*-deacetylation of the sugar moiety undergoing catalysis should yield a potential inhibitor.

Here we report the determination of the stereochemistry of the hydrolysis reaction catalysed by *Serratia marcescens* chitobiase and the competitive inhibition of the enzyme by a series of chito-oligosaccharides selectively deacetylated at the non-reducing end (Figure 1). The results further substantiate the participation of the acetamido group at C-2 of the substrate in the catalytic mechanism of chitobiase and related enzymes.

EXPERIMENTAL

Enzymes

Chitobiase from *S. marcescens* was purified from the periplasmic proteins produced by an *Escherichia coli* clone producing the enzyme, as described previously [9]. Chitinases A1 and D from *Bacillus circulans* were purified as described previously [10].

Determination of the stereochemistry of hydrolysis of chitobiose by chitobiase

p-Nitrophenyl *N*-acetyl-β-D-glucosaminide (PNP-GlcNAc; Sigma, 100 μg) was incubated with 1 μg of chitobiase in 55 μl of distilled water for 5 min. The reaction mixture was analysed by the HPLC method described previously for the determination of the stereochemistry of hydrolysis by chitinases [10].

Preparation of the modified chito-oligosaccharides

Chitopentaose selectively deacetylated at the non-reducing end (N,N^2,N^3,N^4-tetra-acetylchitopentaose, **1**) was prepared by a recombinant *E. coli* strain harbouring the *nodC* and *nodB* genes of *Rhizobium meliloti* as described [11].

Preparation of N,N^2,N^3-triacetylchitotetraose (**2**): the deacetylated chitopentaose **1** (15 mg in 7.5 ml of distilled water) was digested with turkey egg white lysozyme (Sigma; 3 mg) for 30 h at 40 °C. The reaction mixture was subjected to ion-exchange chromatography on Dowex 50 W X 4 eluted first with water to

Abbreviations used: FAB^+, fast atom bombardment (positive mode); EWL, hen egg-white lysozyme; NP-GlcNAc, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide.

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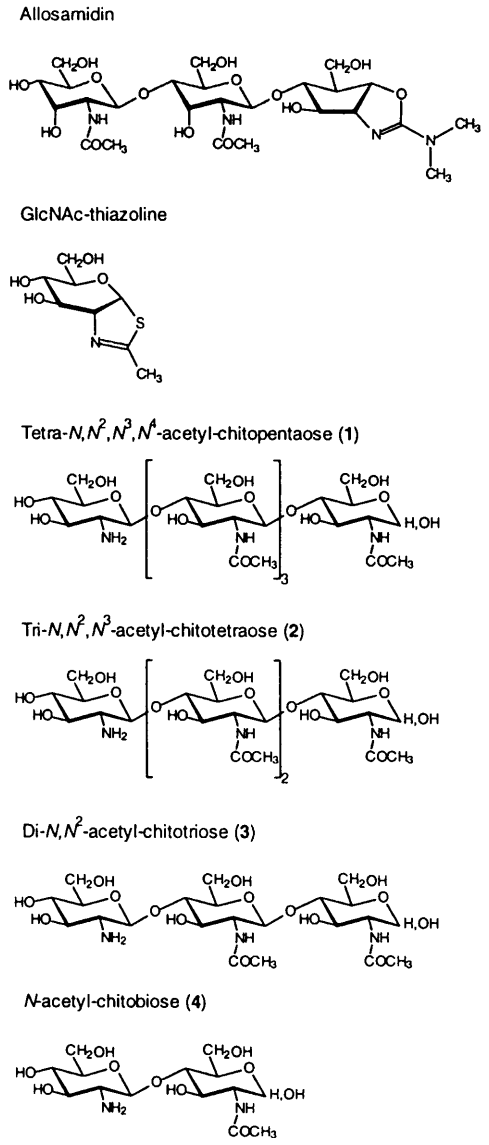


Figure 1 Chemical structure of inhibitors of chitinases and *N*-acetyl-β-D-glucosaminidases

remove the uncharged products (GlcNAc and di-N,N^2-acetylchitobiose) and then with 2% aqueous NH_3 to liberate N,N^2-diacetylchitotriose and N,N^2,N^3-triacetylchitotetraose (**2**). Tetrasaccharide **2** was then purified by reverse-phase chromatography on a C_{18} column (Lichroprep RP-18, 5–20 mm; Merck) eluted with water. $[\alpha]_D^{20}$ −11 ° (*c* 0.5 in water). ^{1}H NMR: δ (300 MHz, ^2H_2O, 30 °C) 5.07 (0.7 H, d, $J_{1,2}$ 2.0 Hz, H-1α), 4.47 (2 H, m, H-1^2, H-1^3), 4.35 (1 H, d, $J_{1,2}$ 8.0 Hz, H-1^4), 3.90–3.20 (23 H), 2.55 (1 H, m, H-2^4), 1.95, 1.91 (9 H, s, $COCH_3$). ^{13}C NMR: δ (^2H_2O) 175.52, 175.41 (CO), 103.76, 102.23 (C-1^2, C-1^3, C-1^4), 95.82 (C-1β), 91.43 (C-1α), 23.08, 22.85 (CH_3). Fast atom bombardment (positive mode) (FAB$^+$) MS: *m/z* 789 $[M+H]^+$, 811 $[M+Na]^+$.

Preparation of N,N^2-diacetylchitotriose (**3**): the deacetylated chitopentaose **1** (15 mg in 5 ml of distilled water) was digested with *B. circulans* chitinase D (0.06 mg) for 16 h at 40 °C and the trisaccharide **3** was isolated directly by ion-exchange chromatography as described for the tetrasaccharide **2**. $[\alpha]_D^{20}$ −8 ° (*c* 0.5 in water). ^{1}H NMR: δ (300 MHz, ^2H_2O, 30 °C) 5.07 (0.7 H, d, $J_{1,2}$ 2.0 Hz, H-1α), 4.49 (1 H, m, H-1^2), 4.39 (1 H, d, $J_{1,2}$ 8.0 Hz, H-1^3), 3.90–3.20 (17 H, m), 2.58 (1 H, m, H-2^3), 1.94, 1.92 (6 H, s, $COCH_3$). ^{13}C NMR: δ (^2H_2O) 175.74, 175.47 (CO), 102.83, 102.31 (C-1^2, C-1^3), 95.85 (C-1β), 91.46 (C-1α), 23.15, 22.60 (CH_3). FAB$^+$ MS: *m/z* 586 $[M+H]^+$, 608 $[M+Na]^+$.

Preparation of *N*-acetylchitobiose (**4**): the deacetylated chitopentaose **1** (15 mg in 5 ml of distilled water) was digested with *B. circulans* chitinase A1 (0.06 mg) for 16 h at 40 °C and disaccharide **4** was isolated by the same procedure as described for the trisaccharide **3**. $[\alpha]_D^{20}$ −5 ° (*c* 0.5 in water). ^{1}H NMR: δ (500 MHz, ^2H_2O, 22 °C) 5.09 (0.7 H, d, $J_{1,2}$ 2.0 Hz, H-1α), 4.61 (0.3 H, d, $J_{1,2}$ 7.0 Hz, H-1β), 4.40 (1 H, m, H-1^2), 3.90–3.45 (8H), 3.40 (1 H, m, H-5^2), 3.30 (2 H, m, H-3^2, H-4^2), 2.60 (1 H, m, H-2^2), 1.94 (3 H, s, $COCH_3$). ^{13}C NMR: δ (^2H_2O) 175.94 (CO), 102.33 (C-1^2), 95.78 (C-1β), 91.49 (C-1α), 23.37 (CH_3). FAB$^+$ MS: *m/z* 383 $[M+H]^+$, 405 $[M+Na]^+$.

Determination of the kinetic and inhibition constants

The enzymic hydrolyses of PNP-GlcNAc were performed at 40 °C in 180 μl of 50 mM phosphate buffer, pH 8.0. Reactions were initiated by the addition of 20 ml of enzyme (4.6 μg/ml). Measurements of initial rates were made at several substrate concentrations, which ranged from 70 to 800 mM. At the concentration of enzyme used, the reactions rates were linear over the selected time course (150 s). All kinetic studies were monitored with a Beckman DU 640 spectrophotometer by measuring the absorbance at 410 nm due to the *p*-nitrophenol released by the enzymic hydrolysis. The kinetic constants K_m and k_{cat} were determined by non-linear regression with the Grafit program (Erithacus Software). For the inhibition studies, incubations were performed in the presence of increasing inhibitor concentrations from 2 to 400 mM. In each case, K_m and V_{max} values were determined as above. The inability of chitobiase to hydrolyse the deacetylated chito-oligosaccharides was tested by incubating oligosaccharides **1–4** (180 μl of a 2 mM solution in 50 mM phosphate buffer, pH 8.0) with 20 μl of chitobiase (46 μg/ml) for 1 h at 40 °C and by assessing the integrity of the substrate by HPLC and TLC (results not shown).

RESULTS AND DISCUSSION

Stereochemistry of the hydrolysis reaction catalysed by chitobiase

The HPLC elution profiles of the products of the hydrolysis of PNP-GlcNAc by the *S. marcescens* chitobiase are shown in Figure 2. The profile in Figure 2(A), a control obtained from a solution of GlcNAc at the anomeric equilibrium, shows that the two anomers are separated on the column. It has been shown previously that the smaller peak, eluted first, corresponds to the β-anomer, whereas the second, larger peak is that of the α-anomer [10,12]. Figure 2(B) shows the HPLC profile recorded after 5 min of hydrolysis of PNP-GlcNAc by the chitobiase. Only the GlcNAc produced appears on the chromatogram, because the remaining PNP-GlcNAc substrate is not eluted in the conditions used. The two anomers of GlcNAc are present but the most abundant peak is that of the β-anomer, indicating that the enzymic reaction operates with overall retention of configuration. The smaller amount of the α-anomer is attributed to the spontaneous mutarotation of the initially formed β-GlcNAc. After 45 min of reaction, the α-anomer of GlcNAc became the most abundant and showed that mutarotation was predominant (Figure 2C). The retaining mechanism of *S. marcescens* chitobiase is likely to be valid for all other members of glycoside hydrolase family 20. Interestingly, a retaining mechanism was previously

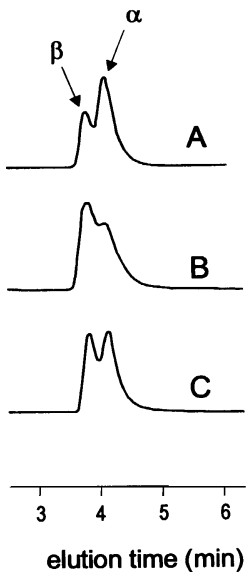


Figure 2 HPLC determination of the stereochemistry of PNP-GlcNAc hydrolysis catalysed by chitobiase

(**A**) Control: authentic GlcNAc standard at mutarotation equilibrium. (**B**) Aliquot of the enzymic reaction analysed after 5 min of incubation. (**C**) As (**B**), but after 45 min of incubation.

demonstrated for three *N*-acetylhexosaminidases from human placenta, jack bean and bovine kidney [12], but the assignment of these enzymes to glycoside hydrolase family 20 is unclear because of a lack of sequence data on these proteins.

Inhibition of chitobiase by chito-oligosaccharides selectively deacetylated at their non-reducing ends

Determination of the kinetic parameters for chitobiase-catalysed hydrolysis of PNP-GlcNAc gave the following values: K_m 56.7 ± 4.3 mM; k_{cat} 111.0 ± 1.7 s^{-1} (means ± S.E.). The hydrolysis of PNP-GlcNAc by chitobiase was competitively inhibited by chito-oligosaccharides selectively deacetylated at their non-reducing ends (Figure 3). The inhibition constants found for oligosaccharides **1–4** were as follows: N,N^2,N^3,N^4-tetra-acetylchitopentaose (**1**), K_i 14.1 ± 1.5 μM; N,N^2,N^3-triacetylchitotetraose (**2**), K_i 50 ± 7 μM; N,N^2-acetylchitotriose (**3**), K_i 167 ± 12 μM; *N*-acetylchitobiose (**4**), K_i 217 ± 16 μM.

The deacetylation of the non-reducing sugar of chito-oligosaccharides not only confers complete resistance to the hydrolytic action by the enzyme but also results in excellent inhibitory properties, providing evidence for the absolute requirement of the acetamido group's participation in the mechanism of chitobiase. It is interesting to note that the affinity of chitobiase for the deacetylated inhibitors increases very significantly with the chain length of the oligosaccharide inhibitors. This suggests that the enzyme might be better described as an exo-chito-oligosaccharide *N*-acetylglucosaminidase with (at least) five sugar-binding subsites rather than a pure disaccharidase, which would be expected to show no increase in affinity for inhibitors when their degree of polymerization was increased from 2 to 5.

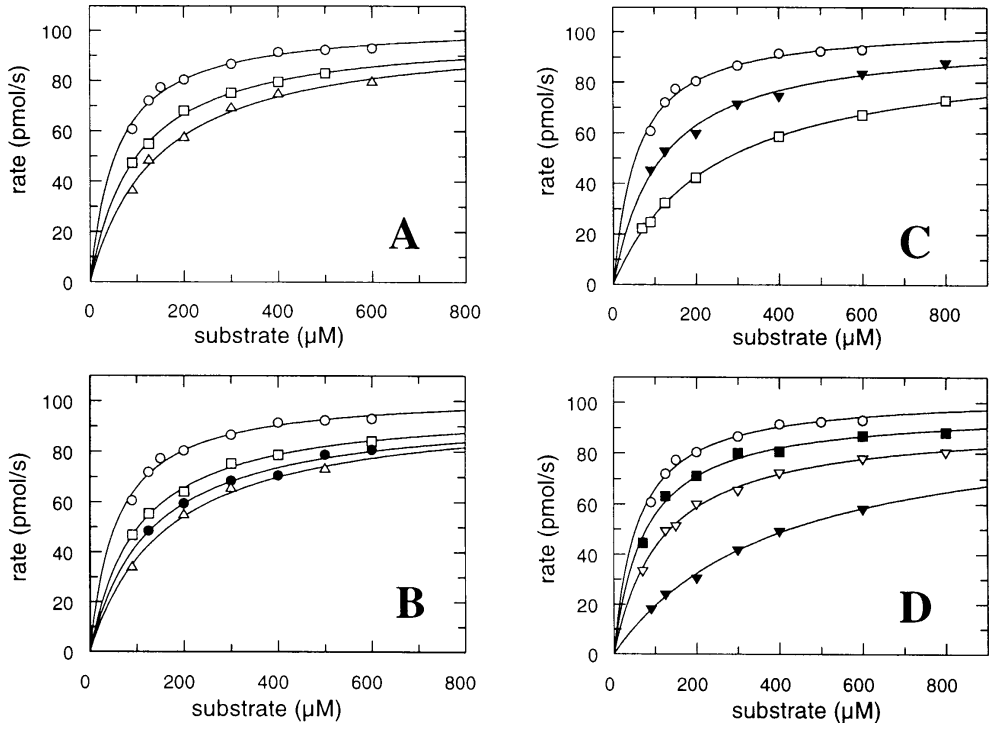
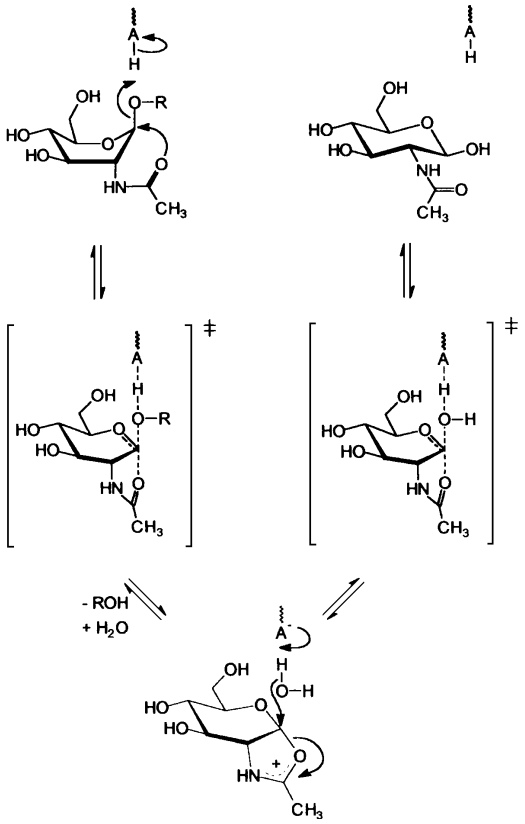


Figure 3 Inhibition of chitobiase by *N*-acetylchitobiose (A), N,N^2-diacetylchitotriose (B), N,N^2,N^3-triacetylchitotetraose (C), N,N^2,N^3,N^4-tetra-acetylchitopentaose (D)

Incubations were performed under standard conditions (see the Experimental section) with increasing concentrations of chito-oligosaccharides: ○, 0 mM; ■, 2 mM; ▽, 20 mM; ▼, 100 mM; □, 200 mM; ●, 300 mM; △, 400 mM.



Scheme 1 Mechanism of chitobiase and related *N*-acetyl-β-D-glucosaminidases

Catalysis is thought to take place via a double-displacement mechanism in which a covalent cyclic oxazolinium ion intermediate is formed and subsequently hydrolysed via transition states having oxocarbenium ion character.

The affinity of chitobiase for the deacetylated inhibitors is lower than that of jack bean *N*-acetylglucosaminidase for GlcNAc-thiazoline [8]. This is consistent with the fact that this compound mimics a preassociated stable reaction intermediate and would be expected to display much tighter binding than a ground-state non-hydrolysable substrate analogue such as the deacetylated chito-oligosaccharides described here.

Mechanistic considerations

Retaining chitinases of family 18 have been shown to hydrolyse GlcNAc-GlcNAc and GlcNAc-GlcN but not GlcN-GlcNAc bonds in partly deacetylated chitin. In contrast, inverting chitinases were found to be able to cleave all these bonds [13]. The conclusion was drawn that retaining chitinases require the acetamido group for their action. Here we show that deacetylation of the sugar residue at the catalytic site results not only in a non-hydrolysable saccharide but also in a new class of inhibitors for this type of enzyme.

A possible interpretation of the inhibitory properties of the deacetylated chito-oligosaccharides is that the removal of the acetamido group of the substrate might induce the loss of interactions involved in transition state stabilization, resulting in a large decrease in the catalytic constants [14]. However, the results that have now accumulated are best interpreted in terms of substrate-assisted catalysis in retaining GlcNAc hydrolases (Scheme 1). The structures of a number of retaining enzymes hydrolysing *N*-acetyl β-D-glucosaminides have been determined recently, notably the family 18 chitinases [15,16], soluble lytic transglycosylase [17,18] and the family 20 chitobiase [2]. A constant feature of all these structures is the absence of a second catalytic group corresponding to a potential enzymic nucleophile. Indeed, the absence of this group initially led to incorrect proposals that some of these enzymes performed catalysis with inversion of the anomeric configuration [16]. What is now becoming apparent is that all these enzymes are likely to utilize a nucleophile donated not by the enzyme but by the substrate itself: the *N*-acetamido group of the GlcNAc residue at the catalytic site. Although documented for well over 40 years [3–5,19], the role of the *N*-acetamido group in the catalytic mechanism of retaining GlcNAc hydrolases has only recently been rekindled by these structure determinations [20]. The absence of an enzymic nucleophile in the chitobiase structure led Tews et al. [2] to propose such a retaining mechanism for the catalytic action of chitobiase. In the present study we have demonstrated that catalysis by chitobiase does indeed result in a retention of the anomeric configuration. The results with deacetylated oligosaccharides therefore provide additional experimental evidence for the role of substrate-assisted anchimeric assistance in the catalytic mechanism of chitobiase and the related family 20 enzymes such as the human hexosaminidases. A powerful additional indication of the widespread occurrence of such a mechanism comes from the strong inhibition of the jack bean *N*-acetyl-β-hexosaminidase by GlcNAc-thiazoline [8] (Figure 1) and from the reactivity of the oxazoline derivative of chitobiose towards a chitinase [21]. At a structural level, overlap of the distorted skew-boat observed in the chitobiase–chitobiose complex, with a similarly distorted sugar observed in a retaining endoglucanase [7,22], demonstrated that the oxygen atom of the *N*-acetamido group of the substrate in the chitobiase structure lies in an equivalent position to the known enzymic nucleophile of the endoglucanase. Finally, it should be mentioned that C-2 acetamido group participation is also found to occur in solution: methanolysis of 2-carboxyphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside proceeds with retention of the anomeric configuration, whereas methanolysis of 2-carboxyphenyl β-D-glucopyranoside yields a mixture of anomers [5].

The mechanism of action of HEWL, widely regarded as a paradigm for enzymic glycoside hydrolysis, still remains a mystery. Unlike all other retaining glycoside hydrolases, which use a covalent intermediate [24,25], either a glycosyl-enzyme or an oxazolinium ion as described here (Scheme 1), HEWL catalysis has been proposed to involve electrostatic stabilization of the oxocarbonium ion intermediate by Asp-52 [26,27]. There have, however, been indications that HEWL might also utilize substrate acetamido participation for catalysis, similar to that described here. Lowe and Sheppard [4] showed that a *p*-nitrophenyl chitobiose derivative in which the C-2 acetamido group of the sugar undergoing catalysis was replaced by an hydroxy group was a very poor substrate for HEWL, implying that acetamido participation was important for catalysis. Indeed the observed decrease in k_{cat} is of the same order of magnitude as that observed with mutations of the supposed oxocarbenium ion stabilizer Asp-52 [28]. An Asp-52→Ser mutant was found to display weak but significant activity towards chitohexaose [29]. There is, however, a significant difference between these early HEWL results and the chitobiase situation. De-*N*-acetylation of the substrate confers total resistance to chitobiase catalysis, whereas the de-*N*-acetylated substrate analogue of lysozyme still remained a viable substrate for HEWL. Structurally, HEWL also differs in that it has a second catalytic residue in Asp-52 that has no counterpart in chitobiase. One possibility,

which could help to explain these apparently contradictory results, is that on either mutation of Asp-52 or deacylation of the substrate, HEWL still retains an appropriate nucleophile and might utilize a 'catalytic rescue', in which the substrate nucleophile might replace the enzymic one and *vice versa*.

In conclusion, the independent lines of evidence brought by chemical considerations [4,5], GlcNAc-thiazoline inhibition [8], structural studies [2,6,7] and the demonstration that chitobiase-catalysed hydrolysis proceeds with a net retention of the anomeric configuration and is inhibited by the deacetylated oligosaccharides, presented here, clearly establish that most retaining GlcNAc hydrolases utilize acetamido group participation during catalysis.

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