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Acetyl xylan esterase-catalyzed deacetylation of chitin and chitosan

Krista L. Morley [a], Grégory Chauve [b], Romas Kazlauskas [a,1], Claude Dupont [c], François Shareck [c], Robert H. Marchessault [b,*]

[a] Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, Que., Canada H3A 2K6
[b] Department of Chemistry, Pulp and Paper Research Institute, McGill University, 3420 University Street, Montreal, Que., Canada H3A 2A7
[c] Institut National de la Recherche Scientifique, INRS-Institut Armand-Frappier, 531 boul. des Prairies, Laval, Que., Canada H7V 1B7

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Abstract

Acetyl xylan esterase catalyzes the hydrolysis of *N*-acetyl groups in chitinous materials of variable degrees of polymerization and acetylation. The influence of substrate accessibility is most notable with substrate of high degree of acetylation (DA). The activity rises sharply as the number of acetyl groups in the substrates decreases and at about 24% DA enzyme activity reaches a maximum. Therefore, based on a multiple-attack mechanism we hypothesize that this maximum represents the ideal acetate microstructure for optimal activity of this enzyme. The enzyme partially deacetylates chitin oligomers DP 2–6 with a plateau in deacetylation observed at DP5. These results show that oligomer length is important for enzyme action. By combining successive alkaline and enzymatic deacetylation a process improvement for production of chitosan is suggested.

Keywords: Chitin; Chitosan; Deacetylase enzyme; Accessibility; Crystallinity; Acetyl xylan esterase; Multiple-attack

1. Introduction

Chitin, one of the most abundant biopolymers, is a structural polysaccharide in arthropod cuticle and skeleton where it constitutes one of the three major components together with proteins and $CaCO_3$. Chitin is a linear polysaccharide of β-(1-4)-linked-2-acetamido-2-deoxy-D-glucose units (Glc-NAc) although approximately 5% of the glucopyranose residues are deacetylated and occur as 2-amino-2-deoxy-β-D-glucopyranose (Muzzarelli, 1977). Chitosan results from the partial or total *N*-deacetylation of the Glc-NAc units leading to β-(1-4)-linked-2-amido-2-deoxy-D-glucose repeating units (Glc-NH_2). Chitin, like cellulose, is insoluble in most solvent systems including water even at acetyl composition of 50% where X-ray crystallinity is still observable, while chitosan is soluble in weak acid solution. Crystalline chitin has a regular hydrogen bonded network, requiring solvents, which either induce interchain repulsions or disturb intermolecular hydrogen bonding for dissolution. Chitosan is used in cosmetics, drug delivery, dermatological products (anti-bacterial properties), agriculture (soil-treatment), textiles (anti-bacterial properties), paper (wet strength), mining (e.g. gold recovery) and water purification. The 'chitosan' label generally corresponds to material with less than 25% acetyl content.

The conventional conversion reaction of chitin to chitosan in strong sodium hydroxide medium proceeds via a micellar reaction whereby alkali-chitin complexes are heterogeneously deacetylated inside and along the fibril according to a pseudo first order reaction (Li, Revol, & Marchessault, 1997; Thor & Henderson, 1940). The reaction usually takes place around 100 °C in a reactor over a period of 15–20 h while prior pretreatments to remove $CaCO_3$, to deproteinize and to bleach the raw material are all part of the overall process. The potential benefits of an enzymatic deacetylation process to make chitosan would truly be in the Green Chemistry realm in terms of energy cost. Deacetylases are well known in the polysaccharide field and recent chitin-related publications using enzymes from *Mucor rouxii*, *Streptomyces lividans* (Caufrier, Martinou, Dupont, & Bouriotis, 2003), *Colletotrichum lindemuthianum* (Tokuyasu, Mitsutomi, Yamaguchi, Hayashi, & Mori, 2000) examine the effectiveness of each deacetylase for the preparation of chitosan. These studies

* Corresponding author. Tel.: +1 514 398 6276; fax: +1 514 398 6256.
E-mail address: robert.marchessault@mcgill.ca (R.H. Marchessault).
[1] Present address: Department of Biochemistry, Molecular Biology, and Biophysics, The Biotechnology Institute, and the Center for Microbial and Plant Genomics, University of Minnesota, 1479 Gortner Avenue, Saint Paul, MN 55108, USA.

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demonstrated the versatility of these deacetylases with respect to substrates.

Acetyl xylan esterases are microbial enzymes that hydrolyze the ester linkages of *O*-acetyl groups at positions 2 and/or 3 of xylose moieties of natural xylan. Such acetyl ester groups are commonly found in hardwoods, cereals and other annual plants. Together with xylanases, β-xylosidases, α-arabinofuranosidases and α-methylglucuronidases, the acetyl xylan esterases are part of the xylanolytic system that is capable of complete biodegradation of xylan (Biely, 1985). Acetyl xylan esterase from *S. lividans* (AxeA) has been cloned, produced and characterized (Dupont, Daigneault, Shareck, Morosoli, & Kluepfel, 1996). The enzyme's substrate specificity and mode of action with full and partially acetylated methyl glycopyranosides has also been investigated (Biely, Côté, Kremnicky, Greene, Dupont and Kluepfel, 1996). Amino acid homology searches indicated that the catalytic domain of *S. lividans* AxeA shares significant similarities with rhizobial nodulation (NodB) protein and chitin deacetylase (CDA) from *M. rouxii*, suggesting that these proteins might be evolutionarily related. Indeed, it has recently been shown that *S. lividans* AxeA possesses CDA activity which hydrolyzes acetamido groups of *N*-acetyl-D-glucosamine in chitin (Caufrier et al., 2003). Both the whole AxeA and the AxeA catalytic domain ($AxeA_{tr}$) displayed CDA activity comparable to that of the CDA from *M. rouxii* when a 60% acetylated soluble chitin with an average molecular weight of approximately 160,000 was used as substrate. This activity was increased 6-fold in the presence of Co^{2+}. Similar results were obtained when *N*-acetyl chitotetraose was used as substrate resulting in 15-fold greater activity for the $AxeA_{tr}$ than for the *M rouxii* CDA, in the presence of Co^{2+} (Caufrier et al., 2003), thus showing the potential of AxeA for enzymatic production of chitosan from chitin. However, enzymatic deacetylation of insoluble chitin remains a challenge due to the substrate accessibility barrier.

The mechanism of action of deacetylases can also influence the level of deacetylation. Chitin deacetylases follow one of three different mechanisms (Grasdalen, Andersen, & Larsen, 1996; Larsen, Skjaak-Braek, & Painter, 1986): *multiple-chain* (random-type) where the enzyme-substrate complex dissociates after each reaction; *single-chain* where the enzyme-substrate complex does not dissociate until every acetyl in a sequence has been hydrolyzed; *multiple-attack* which performs a certain number of successive deacetylations for every enzyme-substrate formed. A multiple-attack process can explain incomplete deacetylation if the enzyme requires a pattern of acetyl substituents to bind and exhausts this pattern after the first deacetylation.

In this paper, we investigate different soluble and insoluble chitinous substrates to determine the CDA potential of *S. lividans* AxeA. These various platforms can be partially deacetylated or fully acetylated. Progressive chemical deacetylation, which requires strong alkali and heat treatment, provides samples with acetyl content in the chitosan composition range. This chemical approach leads to a blocky copolymer made of Glc-NAc or Glc-NH_2 sequences (Li et al., 1997; Li, Revol, & Marchessault, 1999). Interpretation based on the influence of the acetate accessibility and acetyl microsequence is discussed.

2. Experimental section

2.1. Materials

Several chitin and chitosan substrates were used to study enzyme deacetylation. Each type of chitin substrate used in this study is described below while their characteristics are reported in Table 1. Included in Table 1 are the well-known chitin allomorphs where the chains of the α-chitin unit cell are antiparallel while the neighboring chains in β-chitin are parallel aligned.

- The starting purified crab shell chitin (α-Chi) was a gift from ISM Biopolymers Inc., (Granby, Québec, Canada). Its X-ray

Table 1
Characteristics of chitin and chitosan substrates

	Label	DA (%)[a]	Averaged MW per unit (g mol^{-1})[b]	Water soluble
α-chitin	α-Chi	100	204	No
β-chitin	β-Chi	100	204	No
Bleached chitin	B-Chi	100	204	No
Whiskers	W-Chi	100	204	No
Chitin oligomers	Chi-Olig	100	204	Yes
Alkali chitin 92	AC-92	92	200.6	No
Glycol chitin	G-Chi	90	242.8	Yes
Alkali chitin 80	AC-80	80	195.4	No
Alkali chitin 68	AC-68	68	190.2	No
Alkali chitin 61	AC-61	61	187.2	No
Glycol chitosan	G-Chit	40	221.8	Yes
Alkali chitin 31	AC-31	31	174.3	No
Chitosan 24	Chit-24	24	171.3	Yes[c]
Chitosan 17	Chit-17	17	168.3	Yes[c]
Chitosan 8	Chit-8	8	164.4	Yes[c]

[a] Degree of acetylation as analyzed by ^{13}C CP/MAS.
[b] Average of acetylated and non-acetylated units.
[c] Water soluble when first solubilized in weak acidic medium by adding HCl then neutralizing; the insoluble form of the substrate was also tested with AxeA.

diffractogram, infrared spectrum and ^{13}C CP/MAS spectrum are typical of α-chitin. Another purified chitin, also extracted from crab shell, was purchased from Fluka Inc.

- Bleached chitin (B-Chi): starting chitin material (from Fluka) was bleached and deproteinized following the conditions of Nair and Dufresne (2003). After the samples were bleached, the suspension of chitin particles was kept in a 5% KOH solution for 48 h to remove residual proteins. The resulting suspension was centrifuged at 6000 rpm for 10 min followed by drying in an oven at 60 °C for 2 days.
- Beta chitin (β-Chi) was obtained from Kyowa Oil Company, Japan. It was extracted from squid pens, following the same bleaching treatment as performed above.
- Chitin whiskers (W-Chi) were prepared by hydrolyzing the bleached chitin as detailed by Li, Revol, and Marchessault (1996).
- Glycol derivatives: glycol chitosan (G-Chit) was purchased from Sigma. Glycol chitin (G-Chi) was prepared from G-Chit in acetic anhydride and methanol at 40 °C for 6 h (Kurita, Ishii, Tomita, Nishimura, & Shimoda, 1994). This reaction proceeds with selective acetylation of chitosan amino groups. Glycol chitosan was 40% acetylated while glycol chitin was 90% acetylated according to ^{13}C CP/MAS analysis.
- Alkali-treated chitin samples were obtained by special arrangement with Vanson, Inc., (Redmond, WA, USA) (Li et al., 1999). Vanson's deacetylation process consists in treating the chitin raw material in a commercial reactor with strong alkali (50% w/w). During this process aliquots were removed at regular time intervals and after neutralization and washing, partially deacetylated samples were obtained. Alkali chitin substrates are labeled AC-X, where AC represents Alkali-treated chitin and X (in %) corresponds to the degree of acetylation. Temperature applied to this heterogeneous medium ranged between 89 and 97 °C. Four samples, AC-92, AC-80, AC-68 and AC-61 were used in this study.
- Small scale alkali-treated chitin (AC-31) was prepared according to the Vanson procedure (temperature and NaOH concentration) using a pretreated ground and bleached chitin as the starting material (Tsigos, Zydowicz, Martinou, Domard, & Bouriotis, 1999). ^{13}C CP/MAS analysis showed this substrate to be 31% acetylated.
- Chitosan samples with various degrees of acetylation were supplied by Vanson Inc., (8%, labeled Chit-8, 17% (Chit-17), and 24% (Chit-24) acetylated). For experiments involving soluble substrates, chitosans were previously dissolved using HCl (0.1 N) slowly added in aqueous solution under magnetic stirring at 60 °C. When chitosan was completely dissolved, pH was slowly increased with sodium hydroxide 0.1 N to reach a final pH of around 5.3. All final chitosan solutions were prepared to reach a 1% w/w concentration.
- Chitooligosaccharides ranging from DP 1 to 6 were purchased from Seikagaku Inc., (Japan). They are fully acetylated chitin oligosaccharides.

2.2. Enzymes sources

AxeA and $AxeA_{tr}$ were obtained from *S. lividans* IAF43 and IAF934 cultures according to Caufrier et al. (2003); Dupont et al. (1996), respectively. $AxeA_{tr}$ is a truncated form of AxeA lacking the xylan binding domain.

2.3. Enzymatic assay

sp="0.25"/>μL deacetylation reactions were performed using 1 μM of enzyme and either 0.4 mg of soluble substrate or 1 mg of insoluble substrate in phosphate buffer (88 mM, pH 6.2) containing 1 mM ($Co(NO_3)_2$ H_2O). Incubation time was 24 h at 50 °C with agitation (800 rpm). Acetate released was then determined using an existing commercial kit for the detection of acetate (Acetic acid assay, Cat # 0148261, R-Biopharm Inc., Marshall, MI, USA). This acetate release measurement is based on an enzyme-linked assay that forms NADH, which is then detected spectrophotometrically at 340 nm. To analyze the deacetylation reactions in microplate format, the manufacturer's suggested procedure was modified accordingly.

For each substrate, percentage of deacetylation was calculated by dividing the concentration of acetate released in the reaction by the concentration of available acetate groups when the experiment was started. To compare the activity of AxeA and $AxeA_{tr}$ we used equivalent molar amounts of each enzyme in the deacetylation reactions.

2.4. CP/MAS experiments

Solid-state NMR spectra were recorded using a Chemagnetics CMX-300 instrument operating at 75.4 MHz for the ^{13}C nucleus. A recycle delay of 2 s was used. Spectra were accumulated with a spinning rate of 4 kHz. The cross-polarization contact time was 3 ms. 2000–4000 transients were accumulated for most of the experiments. All FIDs were processed with line broadening of 50 Hz and zerofilled to 8 K points before Fourier transformation.

Observations were made on the methyl carbon nucleus, whose chemical shift is around 23.2 ppm.

3. Results and discussion

3.1. Soluble substrates

Recent studies have shown that AxeA and $AxeA_{tr}$ exhibit deacetylase activity on three water soluble chitinous substrates: *N*-acetylated chitosan with a degree of acetylation of 60%, *N*-acetyl chitotetraose and glycol chitin (Caufrier et al., 2003). To further explore the substrate preference of these enzymes, five soluble substrates (Chit-8, Chit-17, Chit-24, glycol chitosan, glycol chitin) ranging between 8 and 90% DA were incubated with AxeA and $AxeA_{tr}$.

Fig. 1 shows the percentage of deacetylation observed after a 24 h incubation with the five substrates as a function of their starting DA. The percentage of deacetylation increases as the

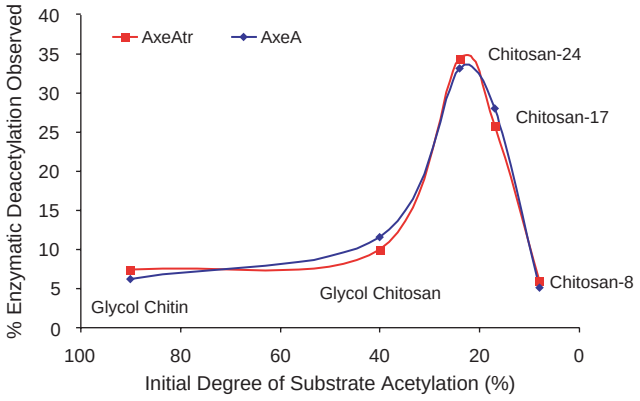

Fig. 1. Enzymatic deacetylation observed for water soluble chitin/chitosan substrates ranging in initial DA (8–90%). Error bars are not shown on the graph for reasons of clarity. Errors were estimated at 5% for the weighing and 5% for the assay measurements.

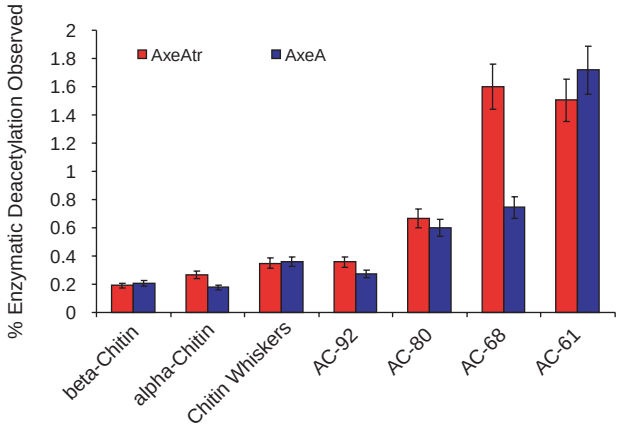

Fig. 2. Enzymatic deacetylation observed for insoluble chitin and alkali-treated chitin substrates.

DA decreases from 90 to 40% (glycol chitin and glycol chitosan, respectively) and reaches a maximum for Chit-24. As the DA decreases from 17 to 8%, a significant decrease in the percentage of deacetylation was also observed. Surprisingly, the substrate with 8% DA was not fully deacetylated either by the AxeA or $AxeA_{tr}$. Since less deacetylation was observed for both 8% DA and 40% DA compared to 24% DA, this suggests the enzyme requires a certain pattern of acetate groups to function efficiently. This might be explained by a *multiple-attack* mode of action, as discussed above.

The writers are aware that other variables may come into play in the deacetylation such as sequence of acetyl groups, number of ethylene oxide moieties in the glycol substitution side chains and solubility. However, our overall results lead us to put more importance on acetyl content than other variables in this paper.

3.2. Insoluble solid substrates

Previous work (Dupont et al., 1996) has reported that AxeA from *S. lividans* is inactive towards crystalline chitin. By comparison, the chitin deacetylase from *M. rouxii* is also inactive towards high molecular weight, insoluble chitin but is more efficient on insoluble amorphous chitin (90.5% DA) (Martinou, Kafetzopoulos, & Bouriotis, 1995). To compare the deacetylase activity of AxeA and $AxeA_{tr}$ with insoluble highly crystalline and less crystalline chitin substrates, the enzymes were incubated with two raw chitins and four alkali-treated chitin substrates (Li et al., 1997).

Fig. 2 represents the histogram of enzymatic deacetylation observed for α-chitin and β-chitin, both pure chitin, as well as for alkali-treated chitin substrates (AC-92, AC-80, AC-68 and AC-61 with decreasing DA). Though the enzymes have low activity on all substrates, the percentage of deacetylation increases as the substrate DA decreases. For AC-68 and AC-61 there is about an 8-fold increase in deacetylation when compared to α and β-chitin, both highly crystalline substrates. Previous studies have shown that the crystallinity of these alkali-treated chitins decreases with time of alkali treatment (Li et al., 1997; 1999).

Chitin whiskers were prepared in order to examine the effect of surface accessibility on the enzyme activity. The experiment was to provide a much higher accessible surface than the crab shells. Even with the same acetyl content, the whiskers were not significantly more deacetylated than the α and β-chitin.

The first three pure chitin samples in Fig. 2 are equally non accessible whereas both enzymes showed more deacetylation activity toward the alkali chitin samples. We conclude that crystallinity is an important barrier to enzymatic deacetylation, which we refer more generally as substrate accessibility.

Even though there is a higher substrate accessibility for the alkali-treated chitins, there still remains some highly crystalline and fully acetylated domains as shown by Li et al. (1997). Thus, after being hydrolyzed in a boiling 3 N HCl solution for 15 min to remove the non-crystalline material, sample AC-61 regained almost the same degree of crystallinity as the original chitin with a significant weight loss of 50%. Since the AC degree of acetylation for the remaining 50% significantly increased after acid hydrolysis, this further indicates that the substrate crystallinity is related to the *N*-acetylglucosamine content and implies a blocky acetate distribution (90% DA) in keeping with the high crystallinity. The loss of the crystalline structure of chitin depends on deacetylation, which prevents the regular intermolecular hydrogen bonding and chain packing characteristic of crystalline α-chitin.

The overall plot of insoluble substrate DA and enzymatic deacetylation is shown in Fig. 3. The three chitosan samples are used as solid substrates and were not previously dissolved in weak acid prior to the experiment. For both enzymes, there is a maximum deacetylation around 24% (Fig. 3), as found with soluble substrates (Fig. 1) where the substrate accessibility factor should be absent. We conclude that this DA where a maximum of deacetylation takes place represents the ideal acetate microstructure in the substrate for optimal activity for this enzyme.

The observed trend in Figs. 1 and 3 is related to substrate accessibility as well as to the reaction mechanism of AxeA and $AxeA_{tr}$ used to deacetylate chitin and chitosan polymers. When

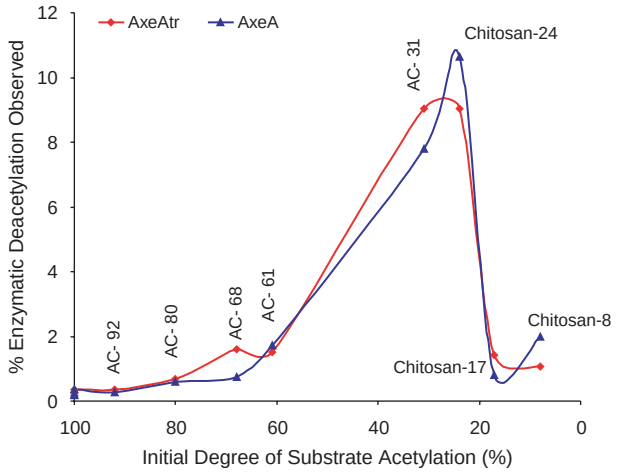

Fig. 3. Enzymatic deacetylation observed for insoluble chitin/chitosan substrates ranging from 8 to 100% initial degree of acetylation Error bars are not shown on the graph for reasons of clarity. Errors were estimated at 5% for the weighing and 5% for the assay measurements.

a specific mode of enzyme action is operative, single-chain, multiple-chain or multiple-attack (Tsigos, Martinou, Kafetzopoulos, & Bouriotis, 2000), the level of deacetylation and also the pattern of acetate removal will be influenced accordingly. For example, CDA from *M. rouxii* uses a multiple-attack mechanism to deacetylate partially *N*-acetylated chitosans, such that a minimum of three successive acetate groups are hydrolyzed before the enzyme binds to another chain (Martinou, Bouriotis, Stokke, & Varumd, 1998). Similarly, the enzyme removes acetyl groups in a processive fashion from the non-reducing end of chitooligosaccharides with a degree of polymerization (DP) greater than two (Tsigos et al., 1999). In contrast, the CDA from *C. lindemuthianum* deacetylates chitooligosaccharides with DP > 1 in a non-processive manner suggesting a random type mechanism for deacetylation (Tokuyasu et al., 2000).

3.3. Mode of action of $AxeA_{tr}$

The $AxeA_{tr}$ enzyme was incubated with chitin oligomers ranging in DP 1–6 to determine the optimum oligomer length for starting enzyme activity. After a 24 h incubation with *N*-acetylglucosamine, less than 1% deacetylation was observed (Table 2); however, deacetylation improved significantly for di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose (19 and 21% deacetylation, respectively). Deacetylation continued to augment with increasing oligomer length such that a plateau of deacetylation at 46% was observed for hexa-*N*-acetylchitohexose. Similar results were also observed for AxeA with DP 1, DP 4, and DP 6 (data not shown); thus we conclude that the xylan binding domain present in AxeA has no influence on the activity or the mode of action of the catalytic domain on chitinous substrates. This deacetylation trend may be a result of the productive binding modes of the chitin oligomers as seen with the CDA from *C. lindemuthianum* where four enzyme subsites interact with GlcNAc residues (Hekmat, Tokuyasu, & Withers, 2003) and influence substrate recognition.

The structure of $AxeA_{tr}$ is unknown, but the X-ray crystal structure of a related peptidoglycan deacetylase (PdaA) from *Bacillus subtilis* was recently solved (Blair & van Aalten, 2004). The central core of both contains a conserved amino acid sequence in carbohydrate esterases called the NodB homology domain (Kafetzopoulos, Thireos, Vournakis, & Bouriotis, 1993). $AxeA_{tr}$ (195 amino acids) and the catalytic domain of peptidoglycan deacetylase (257 amino acids) have a similar amino acid sequence (26% sequence identity) and are both members of the carbohydrate esterase family CE4 (Coutinho & Henrissat, 1998).

This PdaA structure shows a deep substrate-binding cleft long enough to bind at least three *N*-acetylglucosamine units. This deep cleft may account for the higher reactivity of amorphous chitin substrates as compared to crystalline chitin substrates with AxeA. Binding the substrate within its crystalline regions makes binding in a deep cleft almost impossible. The length of this cleft may account for the higher activity of chitin oligomers containing four or more *N*-acetylglucosamine units as compared to chitin oligomers with fewer units (Table 2). The reactivity of a single unit was very low, while dimers and trimers reacted at approximately half of the rate of tetramer, pentamer and hexamer.

4. Conclusions

In summary, the enzymatic deacetylation of chitinous substrates by AxeA is influenced by both substrate accessibility and mode of enzyme action. For all soluble and insoluble chitins/chitosans, AxeA and $AxeA_{tr}$ enzymes exhibit the same deacetylation behavior. They barely act on solid pure chitin whatever its form: native α-chitin and whiskers or native β-chitin. But when chemically treated in strong alkali, chitin is

Table 2
Deacetylation of chitin oligomers (DP 1–6) by $AxeA_{tr}$

Oligomer	Time (h)	Enzyme (μM)	Substrate (mM)	Acetate (mM)	Acetate released (mM)	%Deacetylation observed
DP1	24	1	18.1	18.1	0.14	0.8
DP2	24	1	9.4	18.8	3.64	19.4
DP3	24	1	6.4	19.2	4.01	20.9
DP4	24	1	4.8	19.2	7.26	37.8
DP5	24	1	3.9	19.5	8.58	44.0
DP6	24	1	3.2	19.2	8.77	45.7

50 °C, 24 h, 800 rpm, 1 mM $Co(NO_3)_2 \cdot H_2O$, phosphate buffer (pH 6.2).

partially deacetylated. The longer the alkali treatment, the more efficient the deacetylation of alkali-treated chitins. Chemical acetate removal modifies the crystalline morphology of chitin such that the extent of crystalline barriers decreases. Both enzymes preferentially deacetylate chitosan polymers in the 24% DA range under both homogenous (soluble substrate) and heterogenous (insoluble substrate) reaction conditions. The enzyme partially deacetylates oligomers DP 2–6 with a plateau in deacetylation observed at DP 5. These soluble completely acetylated chitin oligosaccharides probably offer the maximum in suitable binding modes for DP5 and 6 with absence of accessibility constraints.

The enzymatic deacetylation of chitinous substrates by AxeA is potentially a process improvement for alkali-based chitosan production, influenced by substrate accessibility and mode of enzyme action. Overcoming the accessibility barrier for production of chitosan demands severe reaction conditions on alkali-treated chitin after careful pretreatment to purify the starting material. This chemical based process provides a partially deacetylated material. If the second stage is a multiple-attack mode of action by a deacetylase which is effective at 25–30% DA or higher then the tandem chemical/enzymatic approach can be envisaged as a process improvement because of the milder overall processing conditions.

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