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Concurrent enzyme reactions and binding events for chitinases interacting with chitosan oligosaccharides monitored by high resolution mass spectrometry

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ABSTRACT

High resolution nanoelectrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry was used to monitor formation of non-covalent complexes between chitinase B, a family 18 glycoside hydrolase, and hetero-chitooligosaccharides. Besides anticipated productive binding followed by glycoside hydrolysis, additional processes like transglycosylation, non-productive binding of potential inhibitors, and oxidation were detected by analysis of multiple non-covalent enzyme–ligand complexes as well as free oligosaccharide ions. Upon mutation of Asp142, responsible for binding and activation of the *N*-acetylgroup of the -1 sugar for nucleophilic attack on the anomeric carbon, a significant decrease of catalytic activity in the mutant protein was accompanied by drastic changes in oligosaccharide binding preferences and by changed reaction product profiles. The analysis of complex "mixed" enzyme–ligand interactions with unprecedented accuracy and level of detail also provided direct evidence for the occurrence of transglycosylation, leading to the formation of longer oligosaccharides in the reactions with both wild-type ChiB and its D142N mutant. This direct monitoring strategy of distinct types of enzyme–ligand interactions to identify in parallel all products of main and side reactions represents a general approach made possible by MS of ultrahigh resolution and mass accuracy.

1. Introduction

In studying mechanisms of enzymatic reactions, a basic understanding of the enzyme–ligand complex specificity is required. This holds in particular for design of a successful inhibitor and/or tailoring reaction conditions for specific conversions for enzymes acting on multiple substrates and/or having multiple reaction pathways.

For monitoring molecular weights and their changes, binding stoichiometries, relative binding affinities in non-covalent complexes and mapping of the interfacial region of protein–ligand contacts mass spectrometry is offering a direct, accurate and exploratory analytical approach [1–10]. The potential of (nano) electrospray ionization mass spectrometry (nanoESI MS)-based methods for the characterization of non-covalent protein–ligand complexes has been demonstrated in many studies during the past two decades. NanoESI MS has been widely used to detect such interactions of proteins with glycans or glycoconjugates [11,12]. Klassen and co-workers have extensively explored the interaction of different proteins with oligosacharides or glycan-derived ligands in the gas phase with respect to specificity, binding strength and dissociation kinetics [13–17]. Carbohydrate binding to model peptides has been probed by ESI mass spectrometry, recently [18,19].

Chitinase B (ChiB) from *Serratia marcescens* belongs to the family 18 chitinases, capable to interact with and/or to convert polymeric and oligomeric substrates consisting of *N*-acetylglucosamine (A) and glucosamine (D, for deacetylated *N*-acetylglucosamine) [20]. The inhibition of chitinases interfering with chitin turnover is considered as an interesting strategy for combating human diseases as diverse as malaria [21,22], asthma [23] and fungal infections [24–26]. It has previously been shown that some oligomeric D_xA_y oligosaccharides show high-affinity non-productive binding to chitinases [27,28], indicating their inhibitor potential. It should be noted though, that many such D_xA_y oligosaccharides also may be hydrolyzed [29] or participate in transglycosylation reactions (see below).

The outcome of the interaction between chitinases and hetero-chito-oligosaccharides (D_xA_y) is difficult to analyse because the ligands and their products are highly heterogeneous. While it is possible to prepare an oligomer fraction with an uniform degree

Abbreviations: nanoESI-FT-ICR MS, nanoelectrospray ionization Fourier transform ion cyclotron resonance mass spectrometry; ChiB, chitinase B from *Serratia marcescens*; D142N, E144Q, ChiB containing mutations D142N and E144Q, respectively; DP, degree of polymerization; A, N-acetylglucosamine; D, glucosamine (deacetylated *N*-acetylglucosamine); GlcNAc, N-acetylglucosamine; 4-MU-$(GlcNAc)_2$, 4-methylumbelliferyl-β-D-*N*-*N*′-diacetylchitobioside.

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of polymerization [30] and even an oligomer with a homogeneous composition, such as D_2A_3, this oligomer may still contain sequence isomers. In the case of the pentasaccharide D_2A_3 the maximum number of sequence isomers would be 10. In a recent study of non-covalent protein–carbohydrate interactions by MS we were able to develop a novel protocol for identification of a ChiB ligand, a specific pentameric oligosaccharide with the sequence DADAA [27], which was selectively bound by the enzyme out of a complex mixture of hetero-chitooligosaccharides. The ligand was identified by direct MS analysis of the non-covalent enzyme–ligand complex and further ligand analysis by Top Down sequencing, using a nanoESI quadrupole time-of-flight (QTOF) MS instrument. Alternatively, it has been demonstrated that the enzymatic reactivity of endochitinases and chitosanases towards chitooligosaccharides can be monitored by real-time ESI MS assays [31,32]. Analysis of non-covalent protein–carbohydrate interactions by use of mass spectrometric techniques is often complicated by peak broadening caused by adduct formation with both solvent and buffer. Furthermore, mass/charge value changes between the free enzyme and enzyme–ligand complexes are low compared to the total mass. Thus, these types of studies require an instrumentation providing high mass accuracy and resolution.

To create deeper insight into chitinase–ligand interactions and the resulting products, we have exploited the ultra-high resolution of Fourier-transform ion cyclotron resonance (FT-ICR) MS [33] for simultaneous identification of different enzyme–ligand complexes [34–36]. We have studied the ChiB - D_xA_y interaction, where the ligands may exert distinct biological functions, acting as substrates, intermediates, inhibitors or products. The catalytic mechanism of ChiB has been analyzed in several structural and enzymological studies [37,38], but ChiB's binding preferences when interacting with D_xA_y mixtures remained partly unknown, in particular with the chito-oligomeric substrates of DP > 5 [29]. Furthermore, so far, the analysis of catalytic properties has been limited to hydrolysis of glycosidic bonds, whereas transglycosylating activites of chitinases towards mixed D_xA_y oligomers which may very well be anticipated, were unknown on the molecular level [39]. In the present study wild-type ChiB, and its two mutants, E144Q, having ultralow hydrolytic activity due to mutation of the catalytic acid, and D142N, a variant with considerable remaining activity but clearly different binding properties [38,40] were investigated in their non-covalent interactions with chito-oligosaccharides.

Binding and catalytic events were analyzed by studying the molecular composition of oligosaccharide components in the original ligand mixture before and after the enzyme treatment, and by analyzing non-covalent enzyme–ligand complexes at ultrahigh resolution. The results reveal that ChiB is engaged in four different enzyme–ligand interactions, leading to the concurrent processes of hydrolysis, transglycosylation, inhibition (non-productive binding), and lactone formation.

2. Materials and methods

2.1. Protein expression and purification

Wild-type ChiB [28] from *S. marcescens* and its D142N and E144Q mutants were overexpressed in *Escherichia coli* and purified as described elsewhere [38,41]. Enzyme purity was analyzed by SDS-PAGE and found to be >95% in all cases.

2.2. Measurement of chitinase activity

Enzyme activity was determined using the $(GlcNAc)_3$ analogue 4-methylumbelliferyl-β-D-*N*-*N*′-diacetylchitobioside[4-MU-$(GlcNAc)_2$] as a substrate, essentially as described previously [41]. Enzyme concentrations were adapted to the varying activities of the ChiB variants. In an assay, 100 μL of a mixture containing enzyme, 63 μM substrate, 50 mM citrate/phosphate buffer, pH 6.1 and 0.1 mg/mL bovine serum albumin was incubated at 37 °C for 10 min, after which the reaction was stopped by adding 1.9 mL of 0.2 M Na_2CO_3. The amount of 4-MU released was determined using a DyNA 200 Fluorimeter (Hoefer Pharmacia Biotech, San Francisco, CA, USA).

2.3. Hetero-chitooligosaccharide preparation

For preparation of the chitooligosaccharides, the lyophilized product of an enzymatic hydrolyzate of chitosan (degree of acetylation 0.63; 2.16 g; [30]) was dissolved in 180 mL of a 0.05 M ammonium acetate buffer, pH 4.2. The solution was filtered sequentially through a 0.8 μm, and a 0.2 μm cellulose acetate membrane (Schleicher & Schuell), and a 3000 Da cut-off membrane (Amicon), and the filtrate was lyophilized. The residue was subjected to gel permeation chromatography (GPC) on a 5 cm i.d. x 200 cm Biogel P4 column (fine grade; BioRad). The mobile phase consisted of 0.05 M ammonium acetate buffer, adjusted with 0.23 M acetic acid to pH 4.2. Other conditions: flow rate 60 mL × h^{-1}; detector, Shimadzu RID 6A. Fractions of 20 mL were collected, appropriately combined, then concentrated to a small volume by rotary evaporation, and finally lyophylized. Besides small amounts (<5%) of hexamers and octamers (D_2A_4, DA_5, and D_5A_3) the chitooligosaccharide fraction used in this study consists mainly of heptameric oligomers (D_3A_4, and D_2A_5, degree of polymerization, DP7) in various isomeric forms. All these isomeric forms exclusively comprise the sequence AA at the reducing end [30].

2.4. Non-covalent enzyme–ligand interactions preparation

Protein and the multi hetero-chitooligosaccharide solution were mixed and diluted under stirring at 800 rpm and 37 °C to give final concentrations of 3 μM of protein and 150 μM of oligosaccharide in ammonium acetate buffer (40 mM, pH 6.1). The oligosaccharide concentration was calculated by using an average molecular mass of 1313 g/mol. After start of the incubation, aliquots were collected when appropriate and analysed by use of mass spectrometry. Measurements were repeated on different days and mass spectra exhibited a high degree of similarity.

2.5. NanoESI-FTICR MS

Mass spectrometric measurements were performed by use of a Bruker Apex II Fourier Transform Ion Cyclotron Resonance mass spectrometer (FT-ICR MS) equipped with a 9.4 T actively shielded magnet. Gas-phase ions were generated from solutions prepared as described above by nanoESI in the positive ion mode using an Apollo ion source. Typical source conditions for the formation of either uncomplexed gaseous enzyme ions or complexes were: capillary voltage: −670 V and a capillary exit voltage of 130 V. For monitoring free sugars from the incubation assays the capillary voltage was set to −570 V and a capillary exit voltage of 34 V was used to minimize unintended capillary skimmer dissociation of the labile oligosaccharides. The electrospray generated ions were accumulated for 1.0 s in the hexapole located after the 2nd skimmer of the ion source and then transferred into the ICR cell. For the protein and protein/sugar complex ions an ion extraction time of 6000 μs was used while for the oligosaccharide ions this parameter was set to 3500 μs taking into account the intrinsic time of flight effect of the instrument. Trapping was achieved by application of a "sidekick"; trapping voltages were set to 1.0 V for the front electrode and 1.2 V for the rear trapping electrode (for the analysis of the sugar ions these parameters were adjusted to 0.98 V and 1.03 V, respec-

tively). All mass spectra were acquired in the broadband mode in the mass range from *m/z* 200 to 2500 for oligosaccharide-derived ions and *m/z* 3000–5000 for protein and protein/sugar complex ions with 512 kword data points. The time-domain signals were zerofilled once and apodized by a quadratic sine bell function prior to Fourier transformation. However, in some cases signals arising from "Gibbs Oscillations" which result from the truncation of the time-domain ICR signal could not have been removed completely. For all spectra shown, 512 scans (64 for oligosaccharides) were accumulated. The spectra were calibrated externally by use of sodium trifluoroacetate.

3. Results and discussion

3.1. Mass spectrometric characterization of chitinase B from *Serratia marcescens*

Molecular weights of full-size wild-type Chi B and its E144Q and D142N mutants were determined by mass deconvolution [42] of the centroid distribution in a Gaussian shaped envelope with base line separation of isotopically resolved multiprotonated species (Fig. 1). The resulting values were in excellent agreement with the theoretical values calculated from the respective amino acid sequences and corrected for the post-translational removal of the *N*-terminal methionine [43], as well as the formation of one out of two possible disulfide bridges, as observed in the crystal structure [44] (cf. Fig. 1 and Figs. S1 and S2). The accuracy of the experimentally determined molecular masses was typically better than 3 ppm. Oligomerisation of the proteins was not observed.

3.2. Analysis of wild-type ChiB–chitooligosaccharide interactions

The chitooligosaccharide fraction, prepared by gel-permeation chromatography of an enzymatic hydrolysate of chitosan [30], was shown by (+) nanoESI-FT-ICR MS to contain mainly heptamers

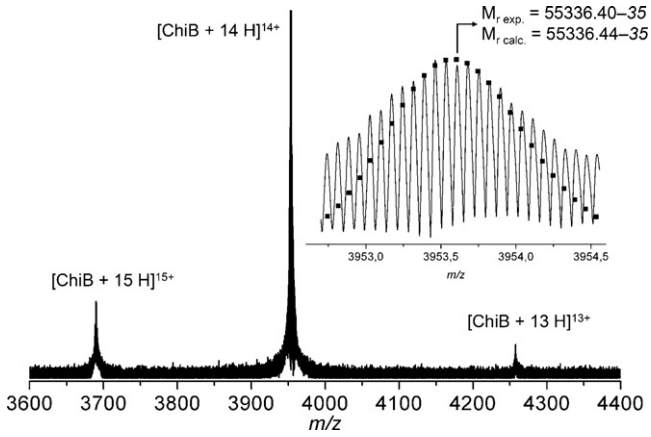

Fig. 1. (+) NanoESI-FT-ICR MS of ChiB from *Serratia marcescens*. The inset shows a mass scale expansion of the isotopically resolved 14-fold protonated analyte species (solid line). Filled squares represent the theoretical abundance distribution of the different isotopomeric species derived from the elemental composition of the protein. The exact masses were determined by comparing the experimentally obtained isotopic envelopes (ion charge state $14H^+$, the most abundant isotopomer) to the theoretical isotopic distributions. The mass of ChiB was determined to be 55336.40-*35* Da [theoretical value 55336.44-*35*; the mass value is followed by the value of the mass difference (in units of 1.0034 Da) between the most abundant isotopic peak and the monoisotopic peak; so "*-35*" indicates that the most abundant isotopomer contains 35 ^{13}C atoms]. Similar experiments (see Figs. S1 and S2 in Supplementary material) yielded masses of 55333.18-*35* Da (theoretical value 55333.34-*35*) and 55333.29-*35* Da (theoretical value 55333.34-*35* Da) for the D142N and E144Q mutant, respectively. Solvent: 40 mM ammonium acetate pH 6.1; protein concentration 3 μM.

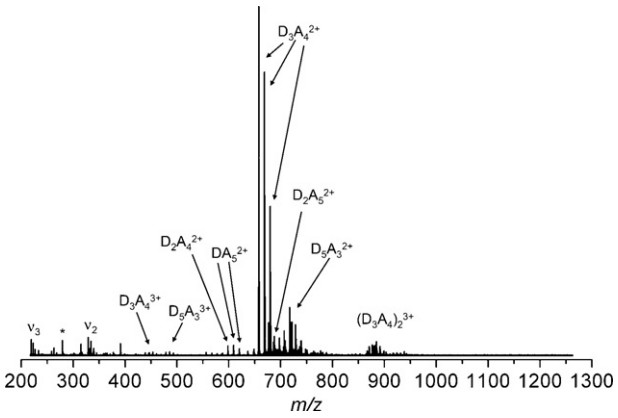

Fig. 2. (+) NanoESI-FT-ICR MS analysis of the multi hetero-chitooligosaccharide substrate solution used in this study (cf. Table S1). The formation of proton and/or cation bound dimers of D_3A_4 results from the high sugar concentration and mild ionization conditions used (low capilliary exit voltage).

D_3A_4, besides several DP6-DP8 components of low intensity (Fig. 2; Table S1).

For MS detection of non-covalent complexes, optimization experiments were conducted which resulted in a system containing 40 mM ammonium acetate, pH 6.1, 3 μM protein and 150 μM of oligosaccharides. The enzyme–ligand mixtures were incubated at 37 °C and MS analyses were conducted after 30 min (all enzyme variants) and 2 h (wild-type and D142N only). For the incubation mixtures comprising either the wild-type or the D142N mutant spectra recorded after 30 min and 2 h were essentially identical, indicating that the steady state was achieved already after 30 min (which was intentional and not surprising considering the very high enzyme concentration). Complete lists of experimental and theoretical masses of sugar species and enzyme–ligand complexes are supplied as supplemental material (Tables S1–S4).

Upon incubation of the sugar fraction with wild-type ChiB, the molecular ion pattern in the high mass range of the spectrum was significantly changed, indicating the formation of distinct non-covalent enzyme complexes with oligosaccharides at different abundances (Fig. 3). The most prominent new ion species was assigned to the complex of ChiB with $D_2A_3[O]$, an oxidized pentasaccharide. This component was not present in the original chitooligosaccharide mixture, which means that it was: (i) generated in a novel type of reaction by ChiB and (ii) trapped by ChiB in a non-productive binding way. Non-productive binding of D_nA_m pentamers to ChiB has been observed before [27,28] and the present observation underpins the potential of such pentamers as inhibitors of the enzyme.

Ions of lower abundance related to complexes between ChiB and hetero-chitooligosaccharides of DP-value 9–12 as D_4A_5, D_4A_6, D_6A_6, were detected as well, indicating that transglycosylation reactions are taking place. In principle, most longer hetero-chitooligomers would be able to bind productively to ChiB [20,29], but such productive complexes would not be observed in the spectra since reactions were run to completion. Thus, the ligands in the observed complexes are most likely trapped as non-productively binding ligands with high affinity, meaning that they act as inhibitors. It is important to note that these elongated oligosaccharides were not present as free ionic sugar species in the nanoESI FT-ICR mass spectrum of the incubation mixture (cf. Fig. 3C and Table S1). The latter observation indicates that all transglycosylation products were enzyme-bound. As indicated in Fig. 2 and Table S1 the formation of low abundant dimeric D_3A_4-derived species, e.g., $[(D_3A_4)_2 + 3H]^{3+}$ ions, were detected in the original sugar mixture. These ionic species can be discriminated from the observed transglycosylation products by the characteristic mass

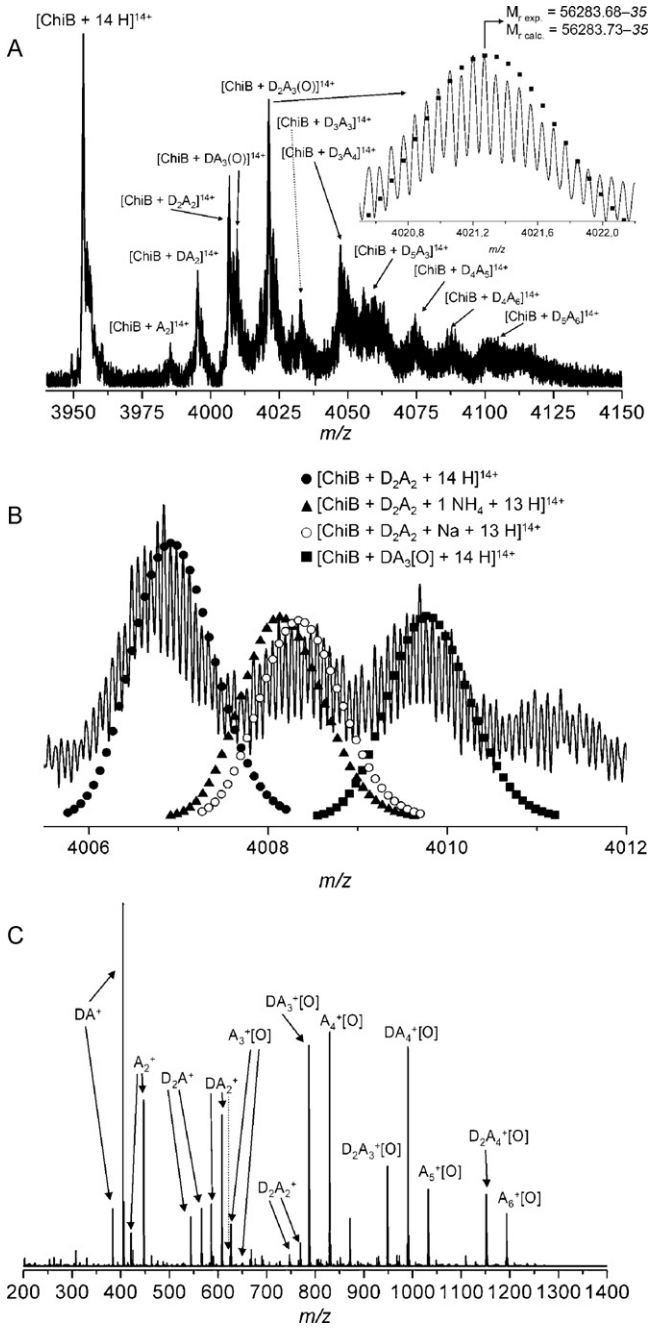

Fig. 3. (+) NanoESI-FT-ICR MS spectra obtained after incubating ChiB wild-type (3 μM) with the multi hetero-chitooligosaccharide substrate (150 μM) solution for 30 min. (A) Spectrum showing free intact protein (ChiB) and non-covalent enzyme–ligand complexes with specific hetero-chitooligosaccharides (+D_xA_y). Only the most prominent complexes are labeled. Lists of the experimental and theoretical masses of all detected enzyme–ligand complexes and of free sugars (panel C) are supplied as Supplementary material (Tables S1 and S2). The inset shows a mass scale expansion of the isotopically resolved ChiB–D_2A_3 complex (solid line) compared with the theoretical abundance distribution of the different isotopomeric species derived from the elemental composition of the complex (filled squares). Note that the apparent "noise" in the spectra is due to the occurrence of numerous variants of the complexes (primarily ammonium adducts) and not to technical limitations of the experiment. (B) Detailed insight into the isotopic distribution in FT-ICR spectrum of four wild-type ChiB complexes with the chitotetrasaccharides D_2A_2 and DA_3[O]. The symbols indicate the theoretical isotopic distribution for the values obtained by calculation. The neutral monoisotopic molecular masses of the most abundant [^{13}C] isotopomer obtained by mass deconvolution are given in Table S2 (cf. Supplementary material). (C) Oligosaccharide products in solution; see Table S1 for a complete list of detected products; [O], oxidized species (cf. text).

shift of 18.0106 u which corresponds to the loss of water upon formation of a novel glycosidic linkage.

Free D_3A_4 oligosaccharides were also not detectable in the lower mass range of the spectrum (Fig. 3C), documenting that they are excellent substrates for the enzymatic hydrolysis by the wild-type ChiB. However, new ionic species corresponding to D_2A_3, D_2A_2, D_1A_2, and A_2 oligosaccharides were generated, which are likely to represent regular hydrolysis products (Fig. 3C). Besides chitooligosaccharides produced by direct hydrolysis, a number of ions were present in the spectra which could not be assigned as simple hydrolysis products. These products, including oxidized A_5 and A_6, result from two subsequent reactions, i.e., transglycosylation and an additional oxidation reaction generating lactones. Sequencing of the oxidized species by MS/MS experiments (data not shown) showed that the lactone functional groups are located at the reducing ends of the oligosaccharides.

We have presently no mechanistic explanation for the formation of these lactones, nor for their escape from hydrolysis. Chitobiono-δ-lactone was shown to bind strongly to subsites -2 and -1 in ChiB, presumably, because the lactone group resembles the transition state of the substrate [45]. It is conceivable that the lactones tend to bind non-productively to ChiB because of the strong affinity of the lactone group for the -1 subsite. It might be argued that the lactone formation represents an artifact due to oxidation processes caused by air or electrochemical redox processes occurring in the ion source. However, ionic species derived from lactones were only detected for two enzyme mutants examined (WT and D142N, vide infra) excluding an non-enzymatic process. Furthermore, oxidized analytes were not observed in the nanoESI mass spectra of buffered heptasaccharide solutions that were kept at room temperature for several days.

It is important to note that the enzyme–lactone complexes give rise to almost isobaric ionic species as compared to other enzyme–ligand complexes containing non-oxidized oligosaccharides. Thus, it would be impossible to detect the lactone complexes in mass spectra generated with detectors of less accuracy and resolution. Besides, such complexes are unaccessible for analysis by alternative analytical approaches, like crystallography and ^{1}H NMR, due to their heterogeneity in solution and their relative low abundance. Thus, the resolving power and mass accuracy of the nanoESI-FT-ICR MS is a prerequisite for the detection of this novel intricate element of protein–carbohydrate interactions and reaction pathways.

3.3. Analysis of D142N–chitooligosaccharide interactions

The mass spectra obtained after incubating the ChiB D142N mutant with the oligosaccharide mixture for 30 min or 2 h were highly similar, indicating that, also in the case of this less active mutant, the reaction reaches equilibrium after 30 min. The resulting spectra (Fig. 4) were distinct from those obtained for the wild-type (Fig. 3). The ions of the native intact free enzyme mutant were of very low abundance, and the spectrum of Fig. 4A thus strongly suggests that almost all enzyme is complexed with a ligand. The ions corresponding to protein–carbohydrate complexes indicate that the bound ligands almost exclusively were D_3A_4 heptasaccharides. Several longer chain transglycosylation products, D_4A_6, D_5A_6, D_6A_6, D_4A_8, and D_7A_7, were also detected in the enzyme–ligand complexes (Fig. 4A; Table S3).

The lower mass range of the spectrum (Fig. 4B) showed highly complex patterns of free oligosaccharides, many of which are likely to be hydrolysis products (Table S1). Additionally, the transglycosylation products, D_4A_6, D_5A_6, D_6A_6, D_4A_7, D_4A_8, and D_7A_7 were present as free oligosaccharides. Like the wild-type enzyme, D142N was able to produce lactones, but at relatively lower abundance.

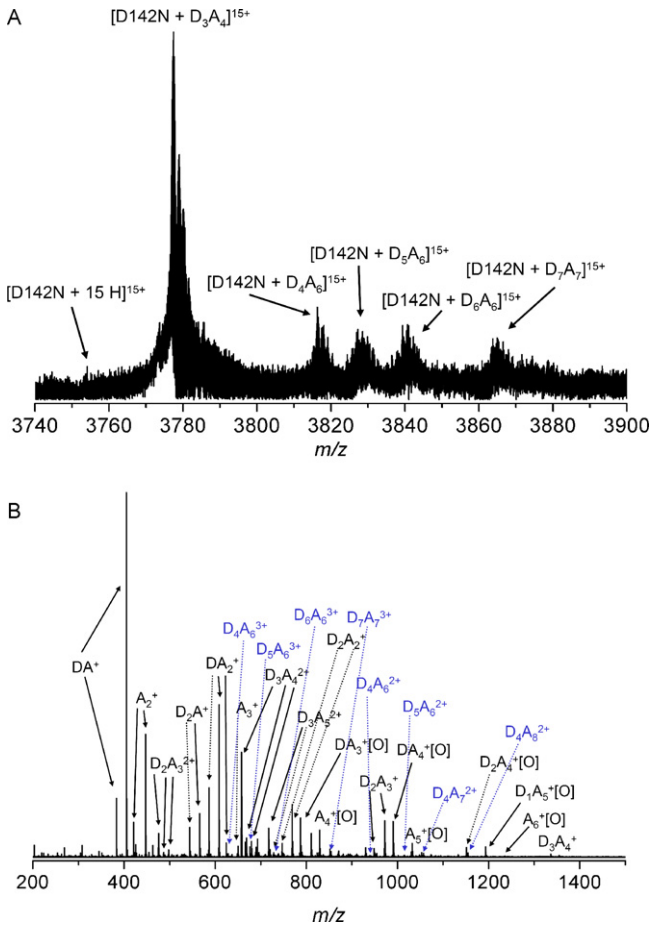

Fig. 4. (+) NanoESI-FTICR MS spectrum obtained after incubating D142N (3 μM) with the multi hetero-chitooligosaccharide substrate solution (150 μM) for 30 min. (A) Spectrum showing free intact protein (D142N) and non-covalent enzyme–ligand complexes with specific hetero-chitooligosaccharides ($+D_xA_y$). Only the most prominent complexes are labeled. Lists of the experimental and theoretical masses of all detected enzyme–ligand complexes and of free sugars are supplied as Supplementary material in Tables S3 and S1, respectively. (B) Products in solution obtained after 30 min of incubation. Blue species indicate transglycosylated products released into solution. [O], oxidized species (cf. text).

From previous work with fluorescent fully *N*-acetylated oligomeric substrates it is known that under the pH conditions used in the present study, the k_{cat} of the D142N mutant is about 60-fold reduced and the K_m about 8-fold reduced, compared to the wild-type [40]. The present data provide much more insight into the mutational effect, showing that the ability of the ChiB enzyme to bind and convert partially deacetylated chitooligosaccharides is strongly affected. The small oligomeric products visible in Fig. 4B show that D142N is still able to catalyze hydrolysis of some heptamers. However, while wild-type ChiB cleaved most of the D_3A_4 isomers, its D142N mutant did not. Instead, a significant fraction of the heptamers binds to D142N in an apparently non-productive fashion as shown in Fig. 4A. The residue 142 is important for catalysis because it distorts the bound -1 sugar and boosts the nucleophilicity of its *N*-acetylgroup [37]. Binding of a sugar to the subsite -1 induces numerous interactions but is in fact likely to be energetically unfavourable due to the distortion of the sugar [46]. Changes in the residue 142 will affect the electrostatics and conformation of this important -1 subsite [40]. One possible explanation for the strong non-productive binding of partially deacetylated heptamers may be that somehow, the -1 subsite in the D142N mutant has a strong preference for binding a D, which is non-productive. It is also possible that the change in interactions between the enzyme and the -1 sugar affects the degree to which suboptimal sugars (i.e., D's) are tolerated in other subsites. Resolving this issue will require advanced crystallographic studies of the enzyme–ligand complexes.

In the mass spectrum depicted in Fig. 4B transglycosylation products of relatively high abundance are present. In preliminary studies with fully acetylated chitooligosaccharides an increased transglycosylation potential of D142N was documented as well (unpublished observations). All in all the data suggest that mutation of Asp142 to Asn had a considerable effect on the ratio between the competing processes of hydrolysis and transglycosylation. Mutants such as D142N may perhaps be used for enzymatic synthesis of hetero-chitooligosaccharides.

3.4. Analysis of the E144Q–hetero-chitooligosaccharide interactions

Upon mutation of the catalytic acid, Glu144, to a glutamine (Q) or alanine (A) the catalytic activity of ChiB is greatly reduced [37,38]. The crystal structure of the E144Q mutant shows no significant changes in the topology of the active site nor in the overall protein structure compared to the wild-type [44]. E144Q was selected as a model for detection of specific enzyme–ligand binding interactions that cannot be observed with ChiB variants of higher enzymatic activity. MS analysis performed after 30 min of incubation confirmed that E144Q is still capable of binding substrate, but largely inactive. The enzyme was found to bind exclusively to two oligosaccharides from the original solution, the D_3A_4 heptaoligosaccharide and the D_5A_3 octasaccharide (Fig. 5A and Table S4 in Supplementary material). The affinity of the E144Q mutant to the D_5A_3 octasaccharide seems to be higher than that to the D_3A_4 heptaoligosaccharide, since the two complexes show approximately equal signal intensities, despite the octamer being present at much lower abundance than the heptamer in the original oligosaccharide mixture.

As expected for an inactive enzyme, in the lower mass range, depicting free oligosaccharides, the D_3A_4 component was dominating, as in the original oligosaccharide mixture (cf. Fig. 2). In addition, very low amounts of hydrolytic oligosaccharides identical to those produced with the wild-type enzyme (D_1A_1, A_2, D_1A_2, D_2A_2) were present (see also Table S1), confirming previous observations that the mutant indeed has some minimal catalytic activity left [37,38]. Transglycosylation products were not observed.

The formation of non-specific protein–ligand complexes under nanoESI conditions has been discussed previously and strategies to discriminate between specific and non-specific association especially for the quantitative evaluation of binding constants have been reported [13,14,47–49]. In the present study, we have examined three ChiB variants differing exclusively by one amino acid residue that exhibit distinct complex formation pattern. Mutation of one amino acid should not alter the overall capability of the different enzymes with respect to non-specific binding. However, the results obtained do not indicate significant formation of non-specfic binding which would result into a random appearance of various complex ions, which is not observed. Furthermore, for the analysis of oligosaccharide ions from the incubation mixtures very mild ionization conditions were applied, i.e., a capillary voltage of −570 V and a capillary exit voltage of 34 V. Therefore, unintended capillary skimmer dissociation leading to formation of fragment ions arising from oligosaccharides is significantly minimized. Consequently, it is much more likely that the data obtained on ChiB–chitooligosaccharide complexes reflect mainly enzymatic reactivity of the different protein mutants rather than being a result of either non-specific binding or unintended fragmentation in the ion source of the instrument.

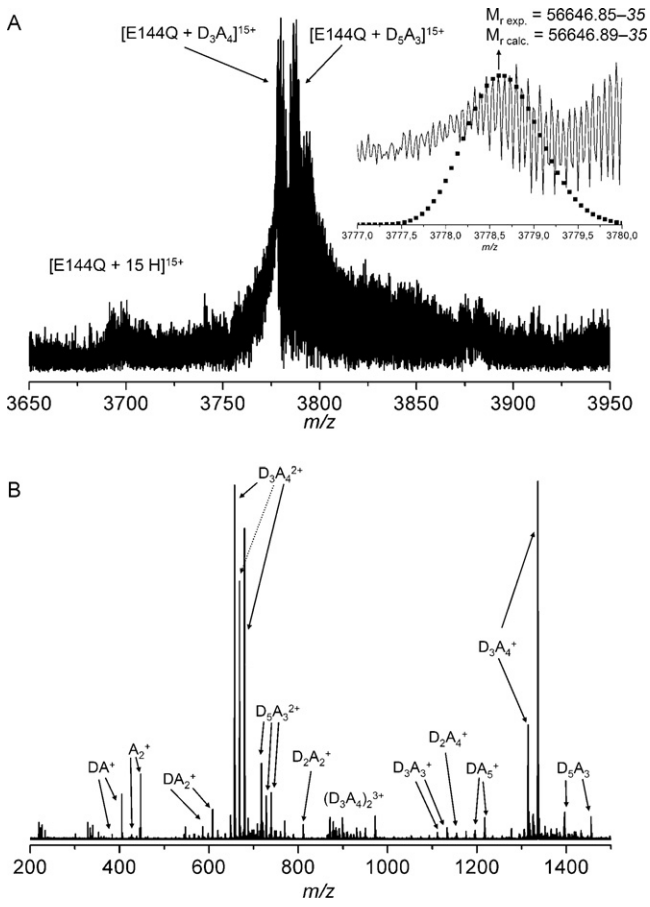

Fig. 5. (+) NanoESI-FTICR MS spectrum obtained after incubating the E144Q mutant (3 μM) with the multi hetero-chitooligosaccharide substrate solution (150 μM) for 30 min. (A) In the enzyme–ligand mass range the split peaks of both complexes are due to the presence of heavily ammoniated species, corresponding to the E144Q_D_3A_4 chitoheptasaccharide or the E144Q_D_5A_3 chitooctasaccharide complex (see also Table S4 in Supplementary material). The inset shows the expanded spectral region (solid line) superimposed with the theoretical isotopic distribution of the [E144Q + D_3A_4 + 14 H + 1 NH_4]$^{15+}$ ions (●) derived from the elemental composition of the protein/oligosaccharide complex to determine exact mass. The neutral monoisotopic molecular masses of the most abundant [^{13}C] isotopomer obtained by mass deconvolution are given in Table S4 (cf. Supplementary material). (B) In the oligosaccharide mass range few products of enzymatic hydrolysis, like A_2, DA_2 and D_3A_3 are present at low abundance, besides the intact components of the starting ligand mixture (Fig. 2). No transglycosylation and oxidation products were detectable (see also Table S1 in Supplementary material).

4. Conclusions

The high advantage of MS analysis over other spectroscopic methods for general explorations of different enzymatic systems has been exploited in this study to analyze binding and enzymatic properties of ChiB, a family 18 chitinase. NanoESI- FT-ICR MS was applied to reveal a multifaceted catalytic picture of a glycoside hydrolase and its mutants in the presence of a complex multi substrate solution. The high resolution and mass accuracy of the FT-ICR MS method was crucial for unmasking the complex landscape of chitinase–carbohydrate interactions. The data indicate the existence of four interaction/reaction pathways: (1) hydrolysis, (2) non-productive binding, (3) transglycosylation, and (4) lactone formation. As illustrated by comparison of the wild-type ChiB and its mutants D142N and E144Q the preference for a certain interaction or reaction in the enzyme molecule can be adjusted by site-directed mutagenesis. To the best of our knowledge, transglycosylation and lactone formation upon interaction of family 18 chitinases with natural hetero-chitooligosaccharide mixtures have not been demonstrated before.

The properties of the D142N mutant revealed in this study are potentially of interest for carbohydrate engineering. The D142N mutant is more prone to form and release transglycosylated products like D_4A_6, D_5A_6, D_6A_6, D_4A_8, and D_7A_7, which, in addition, tend to be of higher DP value than the transglycosylation products produced by the wild-type like D_4A_5, D_4A_6, and D_5A_6.

Interestingly, the hetero-chitooligosaccharides that are observed bound to the enzymes over extended periods of time are likely to represent inhibitors. The D_2A_3 oligomer and its lactone D_2A_3[O] are the most promising in this respect because they are accumulated in large amounts and because they can forms stable complexes with the wild-type enzyme (see also [27,28]). Further studies aimed at unraveling the sequences and the bioactivities of the high-affinity oligomers described above are currently in progress. Simultaneously, the potential of using ChiB, its mutants, and other family 18 chitinases for enzymatic carbohydrate synthesis is being explored further.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.10.031.

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