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Perspectives of Chitin Deacetylase Research

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1. Introduction

Chitin and chitosan are copolymers of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) units linked with β -(1-4) glycosidic bond, where the predominant units are GlcNAc for chitin or GlcN for chitosan in their polymeric chains (Fig. 1). While chitin remained an unused natural resource for a long term, interest in chitosan and chitooligosaccharides (COS) has increased in recent years due to their unique biodegradability, biorenewability, biocompatibility, physiological inertness, and hydrophilicity. Based on these properties, chitosan and COS have been widely and continuously applied in various fields, such as agriculture, cosmetics, water treatment, food industry, pharmaceuticals and biomedicine. Actually, most biological activities of chitosan are strongly dependent on its degree of polymerisation (DP) which defines the molecular mass of the polymers, degree of acetylation (DA) which defines its charge density and pattern of acetylation (PA) which defines the distribution of GlcNAc and GlcN moieties in the chitosan chain.

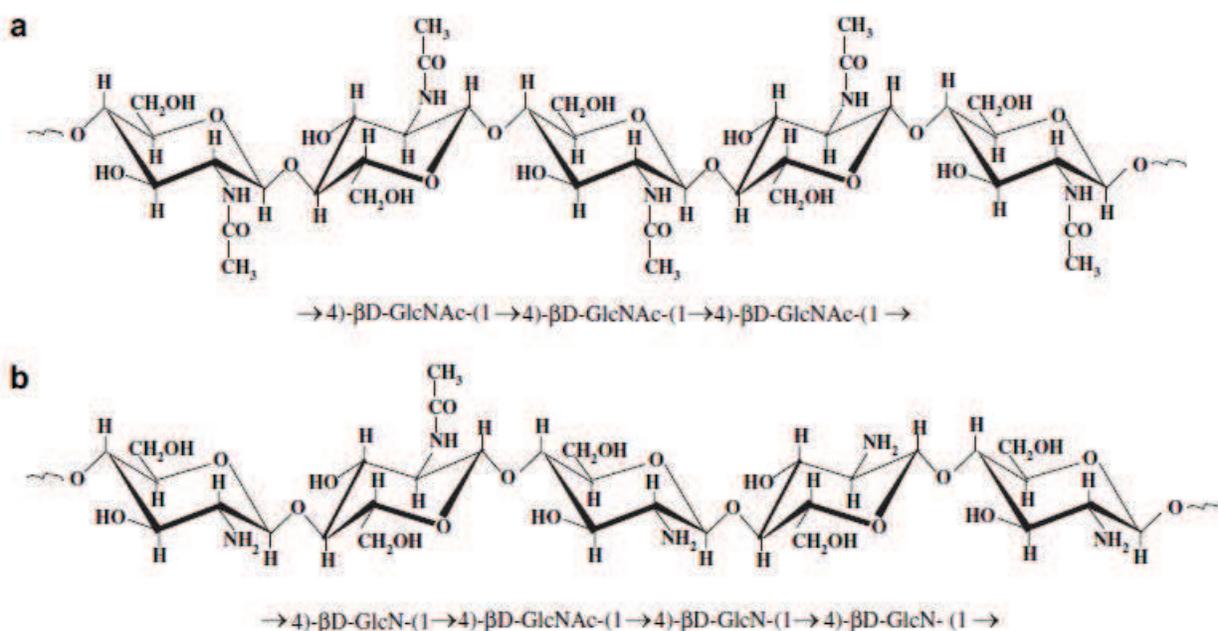


Fig. 1. Structure of chitin (a) and chitosan (b). Cited from Prashanth and Tharanathan (2007).

Commercially available chitosan are generally produced in a heterogeneous process using concentrated NaOH or KOH (40-50%) at high temperatures taking advantage of the speed and low cost of the production process (Peniston and Johnson 1980). The distribution of GlcNAc and GlcN moieties in these heterogeneously generated chitosans shows a random pattern, highly depending on the conditions applied during the deacetylation (Ng et al. 2007). Therefore, although a large quantity of studies on the biological activities of chitosan has been done, variation in biological activities of chitosan is unavoidable and in-depth knowledge on the mode-of-action of chitosan is scarce. The major reason is that most studies are done with badly characterized heterogeneous mixtures of chitosan, especially in its PA.

Alternatively, chitosan can also be prepared enzymatically by harnessing chitin deacetylase (CDA) that was firstly extracted from the mycelium of the fungus *Mucor rouxii* (Araki and Ito 1974). CDA (EC 3.5.1.41) is an enzyme that can hydrolyze the acetamido group in the GlcNAc residues of chitin and chitosan, thus generating GlcN units and acetic acid. It is one of the members of the carbohydrate esterase family 4 (CE-4s), as defined in the CAZY database (<http://afmb.cnrs-mrs.fr/~cazy/CAZY>) (Coutinho and Henrissat 1999). Members of this family share a conserved region in their primary structure, which has been assigned as the "NodB homology domain" or "polysaccharide deacetylase domain". Besides chitin deacetylase, there are several other members in this family, including acetyl xylan esterase (EC 3.1.1.72) (Taylor et al. 2006), chito oligosaccharide deacetylase (EC 3.5.1.-) (John et al. 1993), peptidoglycan GlcNAc deacetylase (EC 3.5.1.-) (Psylinakis et al. 2005) and xylanase A, C, D, E (EC 3.2.1.8). Compared with the chemical method, enzymatic method has the potential to produce some high-quality chitosan products with a well-controlled DA and PA, which is difficult to achieve through a chemical approach.

In this chapter, based on previously published data, a few key issues that exist and hamper the investigation and application of CDA are summarized. Referring to the recently developed techniques or lessons from other research areas, some of our opinions are provided, hoping that would contribute to our in-depth understanding of the catalytic mechanism and biological role of CDA, and further overcoming the hurdles that hamper the application of CDA to produce some high-quality chitosan or COS.

2. Current limitations

Briefly to say, three major challenges need to be addressed. Firstly, the paucity of well characterized CDAs, that are able to result in diverse desired chitosan or COS products, is one issue that cannot be ignored. In a very recent review article, all fungal CDAs reported till now, including their biochemical properties, were summarized and it was found that there are only a very small number of fungal CDAs that have been fully or partially characterized as far (Zhao et al. 2010b). To some extent, exploring novel CDAs with unique properties is of great importance because it is commonly believed that the discovery of more efficient enzymes will enhance productivity and, thus, reduce the cost. Environmental microbes are considered to be the main source of new enzymatic activities owing to their enormous metabolic capability and diversity, much of which currently remains untapped.

Secondly, the scarcity of easy and accurate approaches to establish the structure of enzymatically deacetylated chitosan or COS products is another hurdle to illustrate the action mode and catalytic mechanism of diverse CDAs. To the present, only a little data have been published to explain the action mode and catalytic mechanism of CDAs on substrates of chitin (Martinou et al. 1998) or chitin oligosaccharides (Hekmat et al. 2003,

Tokuyasu et al. 2000, Tsigos et al. 1999), which may at least partially due to the technological limitations.

Thirdly, the scarcity of an efficient method to break the crystalline structure of chitin, that is so recalcitrant that cannot be easily tackled by CDA, is also a key issue to overcome. In this case, two ways may be considered: physical or chemical pre-treatment of chitin substrate or integration of a chitin-binding protein (CBP) along with CDA.

3. Exploring novel CDAs and biological roles

3.1 Novel CDAs

To date, only a small number of microorganisms have been reported to produce CDAs by either identification of CDA encoding genes or determination of CDA activity *in vitro*. By contrast, most of environmental microorganisms remain unexplored but have a strong potential for the discovery of CDAs with unique properties for biotechnological application. It has already becomes apparent that CDA from different sources may differ in their activity, stability, specificity and efficiency. Well-defined COS products have been identified and characterized using novel CDAs from different sources. For instance, CDAs from *C. lindemuthianum* (Tokuyasu et al. 1997) and *Thermococcus kodakaraensis* KOD1 (Tanaka et al. 2004) could exclusively convert GlcNAc-GlcNAc into GlcN-GlcNAc. Besides that, CDAs from *C. lindemuthianum* could also be used for an enzymatic synthesis of GlcNAc-GlcN from GlcN-GlcN (Tokuyasu et al. 1999) and *Thermococcus kodakaraensis* KOD1 could also convert GlcNAc into GlcN (Tanaka et al. 2004). CDA from *Vibrio cholerae* could exclusively convert GlcNAc-GlcNAc into GlcNAc-GlcN (Li et al. 2007), while CDA from *M. rouxii* and *Absidia corulea* could not deacetylate GlcNAc-GlcNAc at all (Gao et al. 1995, Kafetzopoulos et al. 1993).

Finding suitable enzyme candidates depends on efficient and sensitive screening strategies and the greatest possible input of diverse candidate genes and organism. The traditional method of obtaining novel enzymes by cultivation and subsequent screening of pure strains of organisms is a standard and powerful approach. For instance, recently, by the traditional method, two fungi have been isolated from environmental soils and identified to *Mortierella* sp. DY-52 (Kim et al. 2008) and *Absidia corymbifera* DY-9 (Zhao et al. 2010a), both of which can produce an extracellular CDA with a higher enzymatic activity than that from *Mucor rouxii*. In a more recent work, a novel CDA producing strain *Penicillium oxalicum* ITCC 6965 was isolated from residual materials of sea food processing industries and it was also indicated that a following mutagenesis using ethidium bromide and microwave irradiation resulted into a mutant having a two-fold increase in CDA production (Pareek et al. 2011).

Compared with traditional pathways, recent advancements in molecular technologies offer an opportunity to exploit the uncultivable microbes for biotechnological processes (Hess et al. 2011). A metagenomic approach is currently considered the most viable method to search for these elusive enzymes and there has already been some success with the use of several enzymes discovered using metagenomics in the fine chemical and pharmaceutical industries (Lorenz and Eck 2005, Cowan et al. 2005). Metagenomics involves the extraction of the total genetic material from all organisms present in an environmental sample without the need to culture them. The genetic material is then transferred into surrogate organisms to generate a metagenome clone library. To search for specific activities with the metagenome, the surrogate organisms can be screened for particular enzymes, either via DNA sequences or enzymatic functions. Moreover, for functional screening, the number of clones can be

reduced by an initial substrate-enrichment step before metagenomic DNA extraction that corresponds with a particular function. For example, CDA could be isolated from a soil sample by incubating the soil with chitin powder or chitin-containing materials before DNA extraction. This increases the relative number of microbes in the soil that exhibit CDA activity, resulting a higher hit rate during the later stages of the functional screening of clones.

3.2 Biological roles

Besides that, exploring novel CDAs can aid in our understanding the biological roles of CDAs. CDAs have been biologically confirmed to be an essential enzyme required for proper spore formation (Matsuo et al. 2005, Christodoulidou et al. 1996, Christodoulidou et al. 1999), cell wall integrity (Baker et al. 2007), and self-protection through conversion of nascent chitin into chitosan (El Gueddari et al. 2002, Blair et al. 2006). The extracellular CDAs produced by the entomopathogenic fungus *Metarhizium anisopliae* was suggested to own a dual role in modifying the insect cuticular chitin for easy penetration as well as for altering its own cell walls for defense from insect chitinase (Nahar et al. 2004). The mono-deacetylated chitin/chitosan oligosaccharides could further act as a poor substrate for chitinase or chitosanase, by which the pathogenic fungi could successfully avoid the immune recognition by the host (Hekmat et al. 2003, Tokuyasu et al. 2000).

However, there are still many questions that need a further investigation. For example, *Saccharomyces cerevisiae* has two CDA genes that are transcribed only during sporulation (Christodoulidou et al. 1996) and four CDA genes are identified from *Cryptococcus neoformans* (Baker et al. 2007). Why are multiple isoforms of CDA required in the biological process? How do these different CDAs work synergistically? We believe that our knowledge will concomitantly be updated as the exploring of more novel CDAs in illustrating CDA's roles.

Considering the biological roles of CDAs from several plant and human pathogenic fungi, i.e. *C. lindemuthianum* (El Gueddari et al. 2002) and *Cryptococcus neoformans* (Baker et al. 2007), which might protect the pathogen from being attached by the hosts through a modification of the chitin located in the cell wall of the pathogen, a meaningful task will be to design some CDA inhibitors. There is no doubt that both structural and biochemical data will assist the further development of CDA inhibitors (Blair et al. 2006).

4. Catalytic mechanisms

Once we obtain a novel CDA, the next task is to elucidate its substrate specificity and catalytic mechanism. There are many reports about the substrate specificity of CDA from various sources and it was found that most CDAs preferred water soluble chitin substrates only. In addition, the substrate specificity of several selected enzymes classified under Carbohydrate Esterase family 4 has once been examined by Caufrier and co-workers who stated that CDA from *M. rouxii* and both a native and a truncated form of acetyl xylan esterase from *Streptomyces lividans* were active on substrates of glycol chitin, xylan, chitin-50 and (GlcNAc)₄ while inactive on peptidoglycan substrates (Caufrier et al. 2003). As a comparison, there are only very few reports about the catalytic mechanism of CDAs. Actually, there are only two CDAs, whose catalytic mechanisms have been well characterized. In the following part, we will provide a brief introduction of the two catalytic mechanisms of CDAs: multiple attack mechanism and multiple chain mechanism.

4.1 Multiple attack mechanism

In the multiple attack mechanism, binding of CDA on a chitin chain is followed by a number of sequential deacetylations after which the enzyme binds to another chain (Fig. 2A). As an example, the mode of action of CDA from *M. rouxii* has been studied on substrate of partially *N*-acetylated chitosans (Martinou et al. 1998) and *N*-acetylchitooligosaccharides (Tsigos et al. 1999). The enzyme could only effectively deacetylate chitin oligomers with a DP higher than two and the first deacetylation takes place at the nonreducing-end residue of the oligomer. In addition, the extent of deacetylation depends on the length of the substrate. Among chitin oligomers (DP 3-7) tested, (GlcNAc)₄ and (GlcNAc)₅ could be fully deacetylated, whereas the reducing-end residue of (GlcNAc)₃, (GlcNAc)₆ and (GlcNAc)₇ always remains intact with a unknown reason (Tsigos et al. 1999). To explain this, a steady-state kinetic study and structural characterization of the enzyme are required.

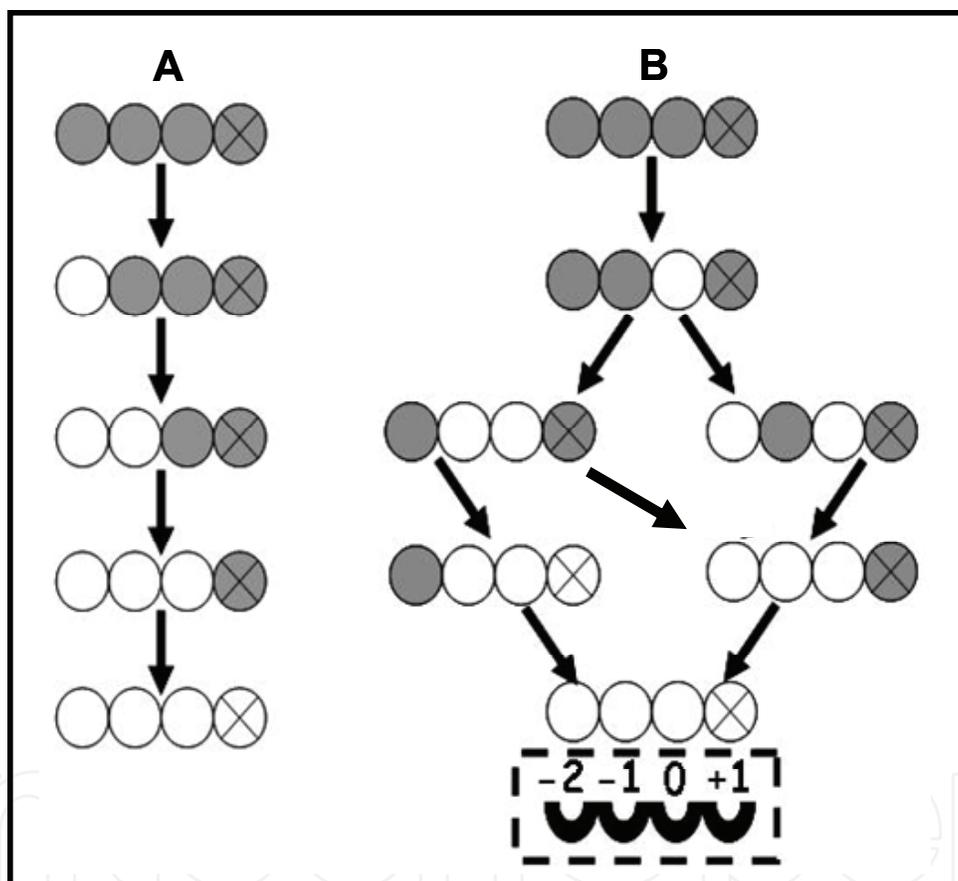


Fig. 2. The pathway of (GlcNAc)₄ deacetylation by an exo-type chitin deacetylase from *M. rouxii* (A) (Tsigos et al. 1999) and an endo-type chitin deacetylase from *C. lindemuthianum* (B) (Tokuyasu et al. 2000, Hekmat et al. 2003). GlcNAc and GlcN are represented by shaded and open circles, respectively. The reducing end residue was indicated by the circle containing X inside. The arrows indicated the sequence by which (GlcNAc)₄ was deacetylated. In Fig. 2A, (GlcNAc)₄ was deacetylated by *M. rouxii* chitin deacetylase from the nonreducing end in a progressive multiple attack mode. In Fig. 2B, (GlcNAc)₄ was deacetylated by *C. lindemuthianum* chitin deacetylase in a multiple chain mode and four subsites are suggested as -2, -1, 0, +1. Among them, only subsite 0 was responsible for the catalysis. Cited from Zhao et al. (2010b).

4.2 Multiple chain mechanism

Compared with *M. rouxii* CDA, a more detailed investigation was carried out on *C. lindemuthianum* CDA. In contrast to the results of *M. rouxii* CDA, the extracellular CDA from *C. lindemuthianum* (ATCC 56676) catalyzed the hydrolysis of acetamido groups according to a multiple chain mechanism, in which the enzyme forms an active enzyme-polymer complex and catalyses the hydrolysis of only one acetyl group before it dissociates and forms a new active complex (Fig. 2B). The enzyme could fully deacetylate (GlcNAc)₃ and (GlcNAc)₄ whereas the reducing-end residue of (GlcNAc)₂ could not be deacetylated (Tokuyasu et al. 1997). In a further study, Tokuyasu and his colleagues carried out a structural analysis of the partially deacetylated products of (GlcNAc)₂₋₆ formed by CDAH (the recombinant non-glycosylated CDA from *C. lindemuthianum*). (GlcNAc)₄ could be exclusively deacetylated to the product of GlcNAcGlcNAcGlcNGlcNAc by CDAH in an initial deacetylation process (Tokuyasu et al. 2000).

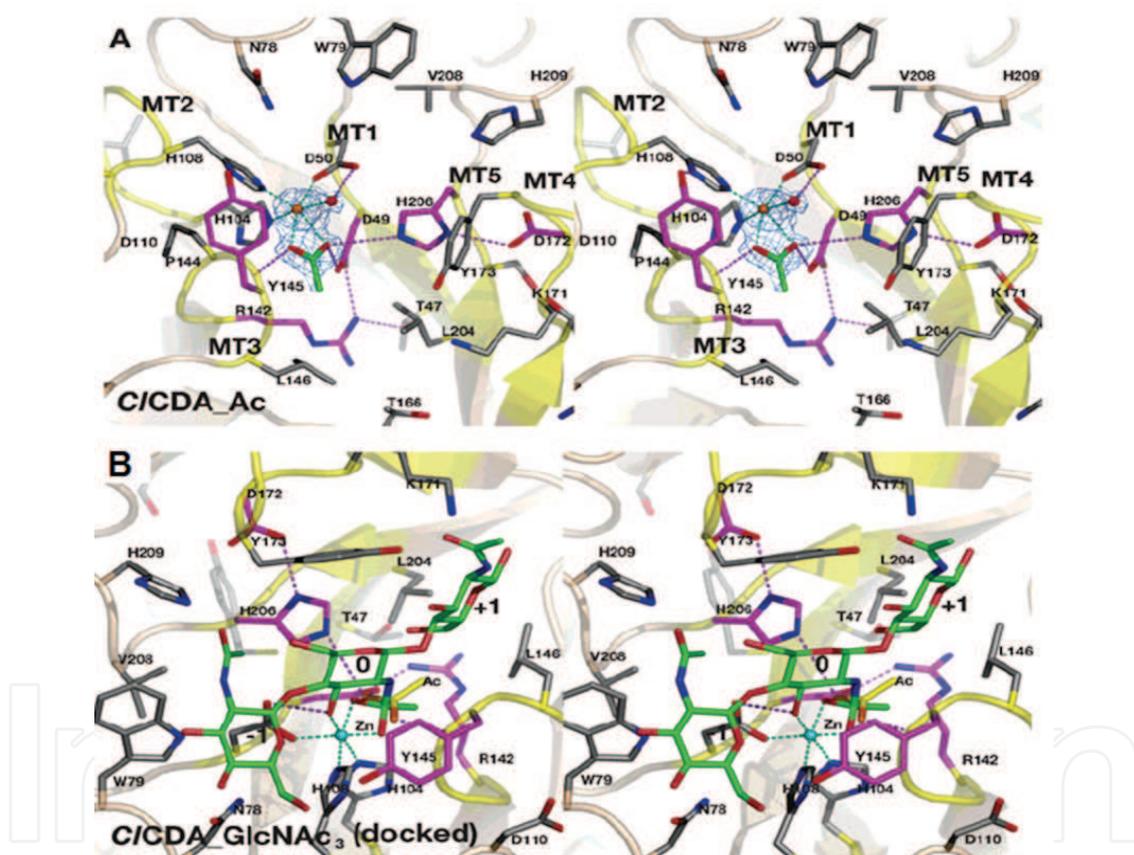


Fig. 3. Details of the *C/CDA* active site. Close up of the active sites of the native *C/CDA* structure in complex with acetate product (panel A, *C/CDA_Ac*) (acetate molecules shown as sticks with green carbons) and a docked GlcNAc₃ complex [panel B, *C/CDA_GlcNAc₃* (docked)]. Ribbon diagrams of the active sites are shown, with conserved side chains shown as sticks with magenta carbons. The experimentally observed acetate is shown as sticks with yellow carbons. GlcNAc₃ is shown as green sticks with subsites labeled -1, 0, and +1. The five conserved sequence motifs (MT1-5) are shown in yellow. Water molecules (red spheres) are also shown. Unbiased $|F_o| - |F_c|$, σ_{calc} maps are shown in blue. Hydrogen bonds are shown in dashed magenta lines (*C/CDA* Tyr145 backbone nitrogen atom is not shown) and zinc-ligand interactions as dashed cyan lines. Cited and modified from Blair et al. (2006).

For a better understanding of the reaction mechanisms, it was proposed that the enzyme has four subsites (-2, -1, 0 and +1). The enzyme strongly recognizes a sequence of four GlcNAc residues of the substrate, and the *N*-acetyl group in GlcNAc residue positioned at subsite 0 is exclusively deacetylated. Among the four subsites, only subsite -2 strongly recognized the *N*-acetyl group of the GlcNAc residue of the substrate, while the deacetylation rate was not affected when either subsite -1 or +1 was occupied with a GlcN residue instead of GlcNAc residue. Afterwards, to confirm the proposed subsites of the enzyme, a full steady-state kinetic characterization of CDAH was carried out (Hekmat et al. 2003). The presence of four enzyme subsites that interact with GlcNAc residues from the non-reducing end to the reducing end of the substrate was experimentally confirmed. The turnover number is independent of *n* and represents the intrinsic rate constant for the hydrolysis of the acetamido group in subsite 0.

The steady-state kinetic parameters for the second deacetylation reaction of (GlcNAc)₄ were also determined using GlcNAcGlcNAcGlcNGlcNAc as the substrate. The results suggest that the mono-deacetylated substrate binds strongly in a non-productive mode occupying all four subsites, thereby inhibiting the second deacetylation reaction (Hekmat et al. 2003).

In a more recent report, the structural data in combination with biochemical data revealed that the catalysis of *C. lindemuthianum* CDA proceeds through a tetrahedral oxyanion intermediate (Fig. 3). It can be proposed that the catalytic base Asp49 abstracts a proton from the water molecule, generating a nucleophile to attack the substrate carbonyl carbon. This produces a tetrahedral oxyanion intermediate, the charge of which is stabilized by the metal Zn and the backbone nitrogen of Tyr145. The pKa-tuned His206 then protonates the reaction intermediate on the nitrogen as it breaks down, generating a free amine and also the acetate product (Blair et al. 2006).

5. Technological aspects

The scarcity of easy and accurate approaches for structural and quantitative analysis of the mixtures of hetero-COS (Fig. 4) is a limiting issue in understanding the catalytic mechanism of CDA. As we know, COS can be divided into homo-COS and hetero-COS, where homo-COS exclusively consist of GlcNAc or GlcN, while hetero-COS are composed of both types of monomer units GlcNAc and GlcN. Analysis of homo-COS by MS is rather straightforward. As there are no isomers, their structures are easily deduced from their molecular mass, which are a multiple of the number of GlcNAc or GlcN residues. The molecular mass of a hetero-COS reveals, however, only its DP and DA, but is silent about structure, since any hetero-COS of the composition (GlcNAc)_m(GlcN)_n can exist as a number of constitutional isomers only differing in the position of the GlcNAc and GlcN residues in the oligosaccharide chain. Characterization of hetero-COS is therefore a formidable structural challenge, in particular when a sample is only available as an intractable mixture of isomers. In the past, an approach harnessing two specific exo-glycosidases in conjunction with HPLC, ion exchange chromatography, NMR spectroscopy or mass spectroscopy (MS) has been used to separate and verify the hetero-COS from enzymatic deacetylation of chitin oligomers by CDA, by which the action modes and kinetic mechanisms of CDA from both *M. rouxii* and *C. lindemuthianum* were elucidated (Fig. 2) (Tsigos et al. 1999, Tokuyasu et al. 2000, Hekmat et al. 2003). However, the above method is very complicated and time-consuming. Besides that, for a complete analysis, a large amount of expensive enzymes and chitin oligomers are required.

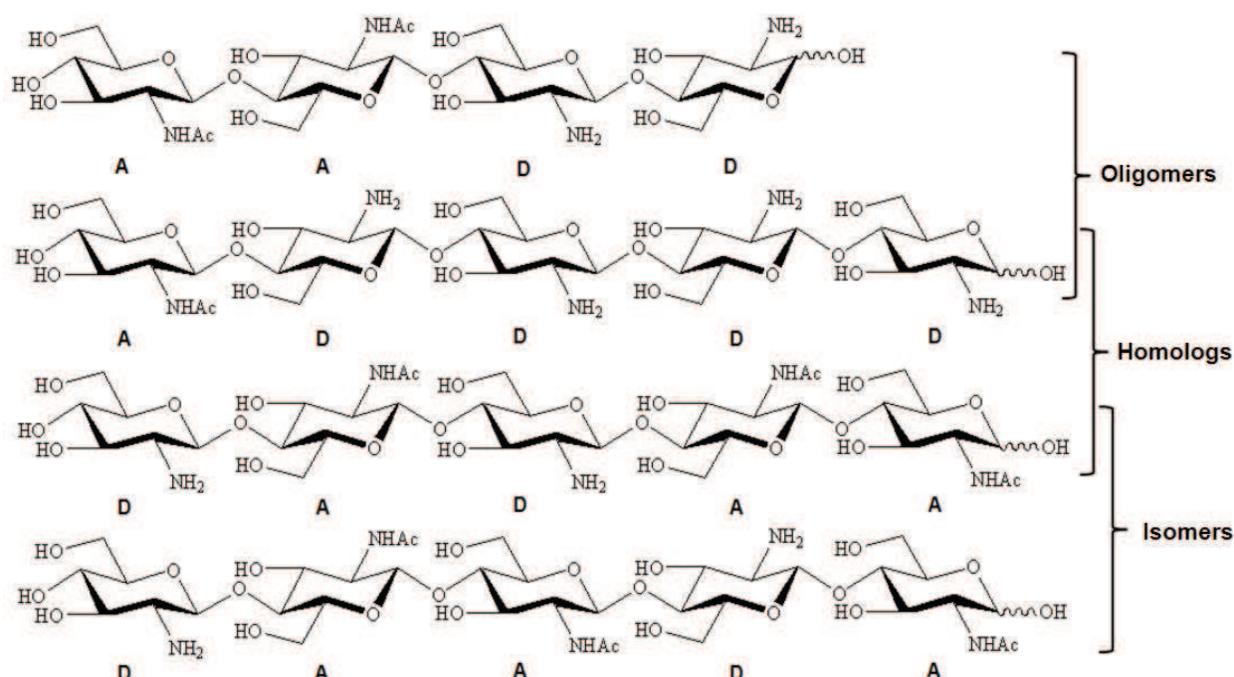


Fig. 4. Examples of heter-COS. Cited from Peter and Eberlin (2010).

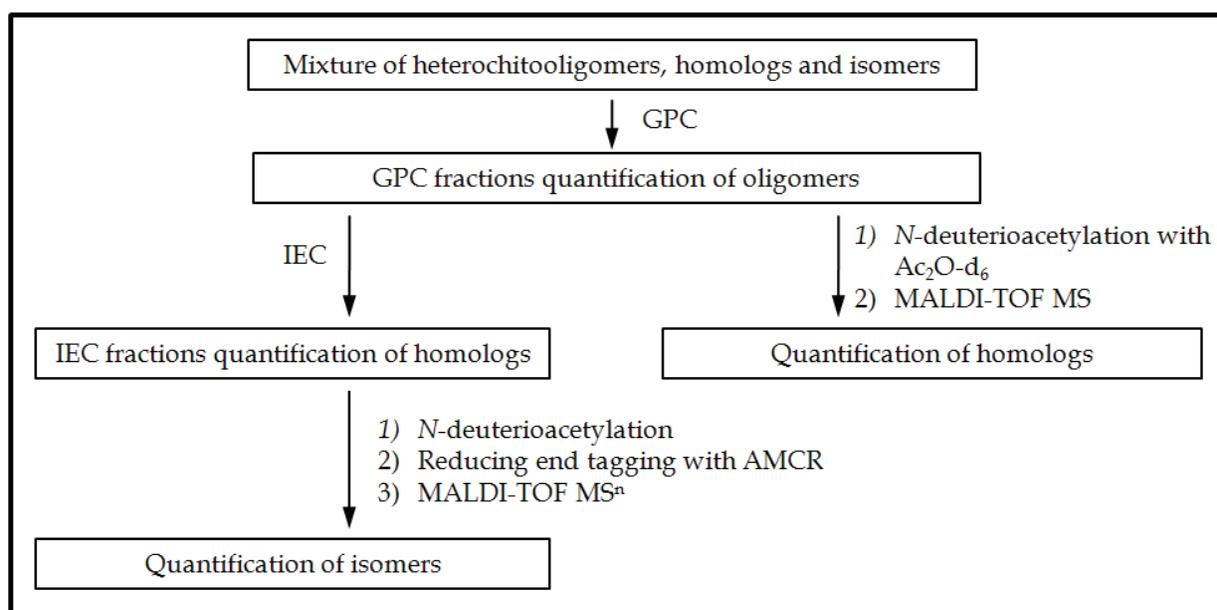


Fig. 5. Summary of the different techniques employed for the quantitative analysis of mixtures containing heterochitooligomers, homologs, and isomers. The first step in any case is gel permeation chromatography (GPC) (on Biogel P4™) resulting in the quantification of oligomers. Ion exchange chromatography (IEC) (on Resource S™) of the GPC fractions allows for the quantification of homologs. Alternatively, MALDI-TOF MS of the GPC fractions after *N*-deuterioacetylation allows for the quantification of homologs. For the quantification of isomers MALDI-LTQ MSⁿ after derivatisation of the desalted IEC fractions with Ac₂O-d₆ (free amino groups) and 3-(acetylamino)-6-aminoacridine (AMCR) (reducing end) is employed. Cited from the Ph.D. thesis of Bahrke (2008).

Compared with the above method, MSⁿ has been shown to be a powerful technique for structural elucidation of hetero-COS (Bahrke et al. 2002, Cederkvist et al. 2006, Haebel et al. 2007, Issaree 2008), which was recently reviewed by Peter and Eberlin (2010). Bahrke summarized different techniques employed for the quantitative analysis of mixtures containing heterochitooligomers, homologs, and isomers (Fig. 5) and the principles of structure analysis of COS by MS (Bahrke 2008). Additionally, in a very recent report, Vijayakrishnan et al. (2011) successfully applied the ESI(+)-MSⁿ into the sequence analysis of six isomers of di-N-acetylchitotetraoses (AADD, ADDA, ADAD, DADA, DAAD, and DDAA, where D stands for GlcN and A for GlcNAc). The advantages of applying this method are the requirement of minute quantities of sample, allowing rapid detection of nano- to picomol quantities of sample, and the possibility to separate the components of even complex mixtures by mass. By taking advantage of MSⁿ, the structure of the mixtures of hetero-COS in the enzymatic reaction process could be easily analyzed so that the action mode of CDAs could be further elucidated. Information of the action mode of CDA will further guide us to understand the biological role of CDA, i.e., why and how the chitin or GlcNAc residues located the fungal or bacterial cell wall are modified by CDA?

6. Dealing with the crystalline chitin

Enzymatic conversion of chitin into chitosan has been investigated for several decades since the discovery of CDA from an extract of *Mucor rouxii* (Araki and Ito 1974), however, a high degree of enzymatic deacetylation is still hard to achieve, mainly due to the insoluble and crystalline nature of chitin. With the ultimate aim of an efficient biotransformation of chitin into chitosan, several studies on improving the substrate properties have been done (Win and Stevens 2001, Beaney et al. 2007). Keeping in mind the enzymatic biotransformation of nascent chitin by CDA in nature, an open and amorphous structure of the chitin is desirable for CDA access to an increased surface area.

Additionally, recent studies on the enzymatic degradation of chitin have provided an important new insight into the mechanism that would allow improved substrate accessibility. Chitin-degradation organisms were found to produce a non-hydrolytic accessory protein, such as the chitin-binding protein (CBP21) from *Serratia marcescens* (Vaaje-Kolstad et al. 2005a, Vaaje-Kolstad et al. 2005b), which promotes hydrolysis of crystalline chitin via non-hydrolytic degradation of the substrate. Such work, which will no doubt expand greatly, begins to shed light on how they may be exploited in chitin deacetylation processes.

6.1 Non-biological modifications

Chitin, without further modification to reduce crystallinity and to open up the solid substrate structure, is found to be a poor substrate for the heterogeneous solid-liquid enzymatic catalysis. Beaney and co-workers found that the solvent and drying method used in modifying the chitins had significant impact on the final efficiency of the enzymatic deacetylation reaction. The most successful modifications through freeze drying of a colloidal chitin suspension increased the degree of enzymatic deacetylation by 20 fold. In addition, several evidences indicated that these processes indeed reduce the crystallinity of the chitin making it easier for the enzymes to access their internal structure (Beaney et al. 2007).

Win and Stevens carried out a series of experiments to improve the enzymatic deacetylation efficiency by various physical and chemical modifications of chitin such as heating,

sonicating, grinding, derivatization and interaction with saccharides (Win and Stevens 2001). It was found that none of those above treatments of the substrate resulted in a more efficient enzymatic deacetylation. In another test, dissolution of chitin in specific solvents followed by fast precipitation by changing the composition of the solvent was not successful either. However, by treating chitin in the specific solvents followed by the fast precipitation, a decrystallized chitin with a very small particle size called superfine chitin could be obtained. This superfine chitin, pretreated with 18% formic acid, appeared to be a good substrate for fungal CDA. Since formic acid is able to dissolve chitin, it must be capable of overcoming intermolecular binding forces in the crystal structure (Win and Stevens 2001).

In our lab, the enzyme activities of two newly screened extracellular CDAs from *Mortierella* sp. DY-52 (Kim et al. 2008) or *Absidia corymbifera* DY-9 (Zhao et al. 2010a) were compared on various chitinous substrates (Table 1). It was found that both crude CDAs preferred water soluble chitin with a degree of deacetylation around 50% (WSCT-50), glycol chitin, chitosan with a DDA between 70% and 90%, while were inactive on other physically or chemically modified alpha-chitin or beta-chitin (Table 1). Although both crude CDAs cannot handle chitin monomer (GlcNAc), comparable with the other fungal CDAs reported till now, it indeed shows enzyme activity on chitin oligomers with a DP ranging from 2 to 7 and their activities increase with the increasing DP (Kim et al. 2008, Zhao et al. 2010a). In a more recent report, Zhao et al. (2011) indicated that a chemical modification of crystalline chitin using phosphate acid could indeed increase the CDA enzyme activity to 3-4 fold, however, the enzyme activity was still very low compared to water soluble chitin.

6.2 Biological modifications

The enzymatic degradation of insoluble polysaccharides, such as chitin, cellulose, glucan, xylan, mannan and starch, is one of the most important reactions on earth. Despite this, glycoside hydrolases attack such polysaccharides relatively inefficiently as their target glycosidic bonds are often inaccessible to the active site of the appropriate enzymes. In order to overcome these problems, many of the glycoside hydrolases that utilize insoluble substrates are modular, comprising catalytic modules appended to one or more non-catalytic carbohydrate-binding modules (CBMs). The most recognized function of these auxiliary domains is to bind polysaccharides, bringing the biocatalyst into close and prolonged vicinity with its substrate, allowing carbohydrate hydrolysis (Boraston et al. 2004).

Among the functions proposed for CBMs, substrate disruption has been a controversial topic, nevertheless information proving this function for some CBMs is convincing. Binding of CBMs to a crystalline substrate leads to polysaccharide chains disorganization and enhancement of substrate availability. Evidence for this substrate disruption, is given by the independent chitin-binding protein CBP21, which belongs to CBM family 33 and is produced by *Serratia marcescens* (Vaaje-Kolstad et al. 2005a). It was suggested that CBP21 promotes efficient crystalline chitin degradation by chitinases through polar interactions that modify the substrate arrangement (Vaaje-Kolstad et al. 2005a). Previously, CBP21 was suggested as a non-catalytic protein, nevertheless, in a recently published elegant work, Vaaje-kolstad and co-workers provided a strong evidence to indicate that CBP21 is actually an oxidative enzyme that catalyzes cleavage of glycosidic bonds in crystalline chitin and its activity could be boosted by ascorbic acid to a much higher level (Vaaje-Kolstad et al. 2010). Their findings not only demonstrated the existence of a hitherto unknown enzyme but also opened up the inaccessible polysaccharide material for hydrolysis by normal glycoside hydrolases.

Harnessing CBMs to bind or disrupt the crystalline polysaccharide is an important point actually not only for glycoside hydrolases but also for some carbohydrate esterases. For instance, a recent report indicated that a cellulose acetate esterase from *Neisseria sicca* SB, which catalyzes the deacetylation of cellulose acetate, contained both a putative catalytic domain and a CBM and the CBM had an affinity for cellulose (Moriyoshi et al. 2010). However, no CDA has been reported to have such CBMs till now.

Therefore, given the excellent performance of CBP21 in promoting crystalline chitin degradation, an efficient enzymatic deacetylation of insoluble chitin could be expected in future through an integration of CBMs/CBPs with CDA by a genetic approach, or an exploring of novel CDA that contains CBMs.

Substrate	Relative activity (%)	
	<i>Mortierella</i> sp. DY-52 CDA	<i>Absidia corymbifera</i> DY-9 CDA
WSCT-50 (Crab, DDA 50%)	100	100
Swollen chitin (DDA 8%)	0	6
Colloidal chitin (DDA 8%)	0	0
Glycol chitin (DDA 8%)	35	19
CM chitin (DDA 8%)	0	13
Regenerated chitin (DDA \leq 1%)	0	0
Crystal β -chitin (DDA 8%, 45 μ m)	0	0
Crab chitin (DDA 8%, 45 μ m)	0	0
Crab chitin (DDA 8%, 150 μ m)	0	0
Crab chitin (DDA 8%, 250 μ m)	0	0
Crab chitosan (DDA 71%, 150 μ m)	49	21
Crab chitosan (DDA 80%, 150 μ m)	7	13
Crab chitosan (DDA 88%, 150 μ m)	9	10
Crab chitosan (DDA 99%, 150 μ m)	0	0

Table 1. Comparison of CDA activity on various substrates

7. Conclusion

Despite major progress in the past decade, the production of pure chitosan or COS with defined DP, DA and PA is still a challenge. To overcome this problem, exploring some novel CDAs with unique properties, understanding their catalytic mechanisms, and modifying either chitinous substrates or CDAs for an efficient catalysis should be focused in the future.

8. Acknowledgment

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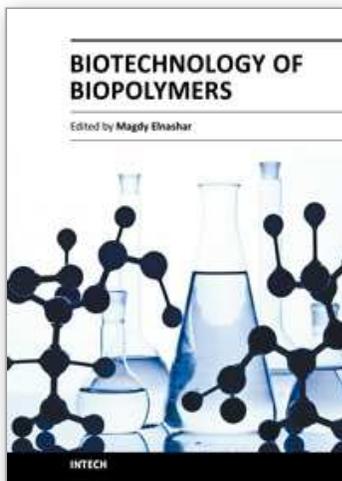
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The book "Biotechnology of Biopolymers" comprises 17 chapters covering occurrence, synthesis, isolation and production, properties and applications, biodegradation and modification, the relevant analysis methods to reveal the structures and properties of biopolymers and a special section on the theoretical, experimental and mathematical models of biopolymers. This book will hopefully be supportive to many scientists, physicians, pharmaceuticals, engineers and other experts in a wide variety of different disciplines, in academia and in industry. It may not only support research and development but may be also suitable for teaching. Publishing of this book was achieved by choosing authors of the individual chapters for their recognized expertise and for their excellent contributions to the various fields of research.

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