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Heterologous production and characterization of chitin deacetylases with chitin binding domains

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Summary

Chitosan, a linear polysaccharide composed of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN), is commercially produced from chitin by deacetylating shrimp-chitin chemically and used for a wide range of applications, including medicine and plant protection. The term chitosan refers to a group of oligomers and polymers that differ in their degree of polymerization (DP), their degree of acetylation (DA) and their pattern of acetylation (PA), the last one being the sequence of GlcNAc and GlcN units. The parameters DP and DA influence the physicochemical properties and biological activities of chitosans; therefore, it is proposed that this is also true for PA. In nature, chitosan is typically generated from chitin by enzymes that catalyze the hydrolysis of the acetyl group, named chitin deacetylases (CDAs, EC 3.5.1.41). CDAs occur in insects, fungi and in chitin-degrading bacteria where they are part of the chitin catabolic pathway. In fungi, they function either in the cell wall formation in the case of zygomycetes, or in the modification of the fungal cell wall chitin, later in development. For plant pathogenic fungi, CDAs play an important role during infection by influencing the plant-pathogen interactions. The conversion of the fungal cell wall chitin into chitosan protects the polymer from plant chitinase and additionally, the plant response reaction to chitin oligomers is reduced by a partial deacetylation.

CDAs have been isolated directly from fungus or have been heterologously expressed for biochemical characterization. CDAs investigated so far have been grouped in two types: intracellular CDA from zygomycetes acting processively and extracellular CDA from other phyla with a non-processive mode of action. Although the mode of action has only been solved for one CDA of each group, it is conceivable that the use of CDA results in chitosan products with a non-random PA and therefore different from the chitosan chemically produced. Despite their name, CDAs act on the insoluble crystalline chitin to a minimal extent only, depending on the crystallinity of the chitin preparation, because the deacetylation is limited to the surface of the chitin particles. Scientific research is aiming for novel CDAs producing chitosans by hydrolyzing crystalline chitin and thereby generating water-soluble chitosan with defined, non-random PAs.

The topic of this thesis is a special type of CDAs differing from biochemically characterized CDAs, because they possess a multi-domain structure containing additional chitin binding domains (CBDs). These domains occur in chitinases as well and are described to enhance the binding towards

crystalline chitin. In order to characterize novel CDAs, two candidates were selected for heterologous expression, one from *Botrytis cinerea* (BC95) and one from *Podospora anserina* (PaCDA). BC95 was identified as one of six putative CDA genes in the *B. cinerea* genome and expression studies for these genes during conidia germination revealed an upregulation of BC95 in fructose containing medium. Heterologous production of BC95 revealed challenges that were most probably due to the large amount of disulfide bonds (14) in the CBDs and the catalytic domain of the enzyme. Using *Schizosaccharomyces pombe* and *Escherichia coli*, BC95 was not produced at all, whereas the production in *Hansenula polymorpha* was partly successful. Despite the secretion signal, the presence of BC95 was limited to the intracellular fraction, because BC95 was not completely processed and not secreted by *H. polymorpha*. The low mobility during SDS-PAGE analysis with multiple bands appearing above 75 kDa suggested that the MF-α1 pre-propeptide was not cleaved and that the protein was highly glycosylated. CDA activity tested on different chitinous substrates was not demonstrated; thus, it remained unclear if BC95 is a CDA or if the inactivity was caused by missing or incorrect processing.

In contrast to BC95, PaCDA was successfully produced in *H. polymorpha,* being the first CDA containing CBDs for which CDA activity was demonstrated. PaCDA was identified by a protein sequence alignment of the catalytic polysaccharide deacetylase domain (PDD) sequences and comparing biochemically characterized CDAs to sequences of putative CDAs available in databases and possessing a PDD/CBD multi-domain structure. PaCDA was purified from the culture supernatant and biochemical characterization of PaCDA revealed similarities to the well-characterized *Colletotrichum lindemuthianum* CDA, for example in the presence of a cofactor (Zn^{2+}), the pH (8.5) and temperature optimum (55°C). The role of the CBDs for PaCDA was investigated by constructing three deletion variants lacking the N-terminal (ΔN), the C-terminal (ΔC) and both domains (ΔNC). The production and purification of PaCDA-ΔC and PaCDA-ΔNC failed, hence, only PaCDA-ΔC was compared to the full length enzyme. The comparison revealed slight differences in the temperature optimum and mode of action on chitosan, but the same activity on colloidal chitin. The amounts of acetate released by PaCDA using insoluble chitins demonstrated that the deacetylation was limited to GlcNAc units accessible on the surface of the chitin particles.

In conclusion, the CBDs might help the PaCDA to stay close to the substrate for a fast deacetylation of accessible GlcNAc units, but a disruption of the chitin crystal and the conversion to water-soluble chitosan was impossible. To investigate the mode of action of PaCDA, chitosan was used as substrate and the enzyme was found to be non-processive; furthermore a specific non-random PA was produced. This PA was analyzed by enzymatic/mass spectrometric fingerprinting as well as ^{13}C-NMR and contained less GlcNAc units next to each other than a chemically produced chitosan with the same DP and DA. Preliminary comparative experiments on the biological activity of these two chitosans differing only in PA revealed no differences for the oxidative burst assay using suspension culture rice cells, but differential anti-microbial activities towards *Fusarium graminearum* and *Lactobacillus plantarum.* To my best knowledge, this non-random, alternate-dominated PA created by PaCDA in this study is the first described so far and represents a novel characteristic for further investigations on chitosan properties.

1. Introduction

1.1. Chitin and Chitosan

In the past decades an ongoing trend to natural products became apparent and induced the demand for sustainable and renewable resources. Polysaccharides are widely used and one of the most abundant biomolecules present in all kingdoms, which include cellulose and pectin from plants, carrageenan and alginate from algae, xanthan produced by bacteria as well as chitin and chitosan from arthropods or fungi. Chitosan is the deacetylated derivative of chitin, which is a widely spread polysaccharide initially discovered in fungi [Muzzarelli et al., 2012] and commercially isolated from shrimp shells [Vázquez et al., 2013]. 60 years after its first discovery [Kreger, 1954], chitosan attracted much attention nowadays, as seen by an exponentially increasing number of publications on chitosan oligomers and polymers [Aranaz et al., 2009]. Scientific research addresses the broad application spectrum of chitin and chitosan based on their biodegradability, biocompatibility and biological activities as natural and biofunctional polymers [Ravi Kumar, 2000].

Chitosan-derived dietary supplements including glucosamine are the highest volumes of chitosan products consumed worldwide [Sandford, 2002]. Chitosan is also applied in large amounts for other functions, like wastewater treatment [Bhatnagar and Sillanpää, 2009], plant protection [El Hadrami et al., 2010; Hadwiger, 2013] and food preservation [Aider, 2010], especially in Asia [Kurita, 2006]. In contrast, medical applications of chitosan are explored intensively for profitable products [Aam et al., 2010; Park and Kim, 2010], including nanomaterials [Shukla et al., 2013], wound healing [Jayakumar et al., 2011; Muzzarelli, 2009] and drug delivery [Hu et al., 2013], also with chemically modified chitosans [Zhang et al., 2010]. Despite the wide range of chitosan application, we need to progress in understanding the biological mechanisms of chitosan, because they are based on the different properties of this saccharide molecule family. The relation of the physicochemical properties of chitin and chitosan to their biological properties and applications are comprehensively discussed [Aranaz et al., 2009].

1.1.1. Structure and properties

Chitin and chitosan are linear polysaccharides consisting of β-1,4-linked units similar to cellulose. Chitin is composed of N-acetylglucosamine (GlcNAc or A) units only, but in the case of chitosan, glucosamine (GlcN or D) units are present as well. Another definition is based on the solubility; as a chitin may not be 100% acetylated, it is still insoluble due to strong intermolecular hydrogen bonding

[Gardner and Blackwell, 1975], while chitosan with up to 70% GlcNAc units is soluble in weak acids [Roberts, 1992]. Solubility [Pillai et al., 2009], solution properties and polyelectrolyte effect of the protonated amino group are important qualities for the usability [Anthonsen et al., 1993]. These properties are influenced by parameters like the molecular weight and the degree of acetylation (DA) of chitosan that have to be considered during the production. The DA can be regulated by a timed or stepwise chemical deacetylation of chitin [Sannan et al., 1976] or more precisely by a complete deacetylation to a DA about 0% [Domard and Rinaudo, 1983] and a subsequent reacetylation with an exact amount of acetic anhydrite calculated for the intended DA [Lavertu et al., 2012; Vachoud et al., 1997]. The size of the molecules can also be modified by chemical or enzymatical depolymerization [Jeon et al., 2000] and is described by the degree of polymerisation (DP) that is the number of units (Fig. 1A).

1.1.2. Biological activities

In many publications it has been reported that the DP and DA are greatly influencing the biological activities, so only some examples can be included here. A polymeric chitosan with a medium DA of 35% caused the strongest resistance reactions when injected to wheat leaves [Vander et al., 1998], while the anti-fungal activity of chitosan oligomers were the highest with the lowest DA [Oliveira et al., 2008]. Both values influenced the callose formation of *Catharanthus roseus* cells indicating that the negative charges of the amino groups are involved in interactions to the cell membranes [Kauss et al., 1989]. It also has become evidence that the properties of chitosan not only depend on DA and DP, but also on source and preparation method [Qun et al., 2007; Tolaimate et al., 2003]. For applications of chitosans to plant or human cells and tissues, the presence of chitinases usually possessing a specific cleavage activity has to be considered. Thus, the hydrolytic activity and the resulting products depend on the sequence of units present in the polymer (Fig. 1B). Hence, a third chitosan parameter of becomes important for bioactivity that is the pattern of acetylation (PA).

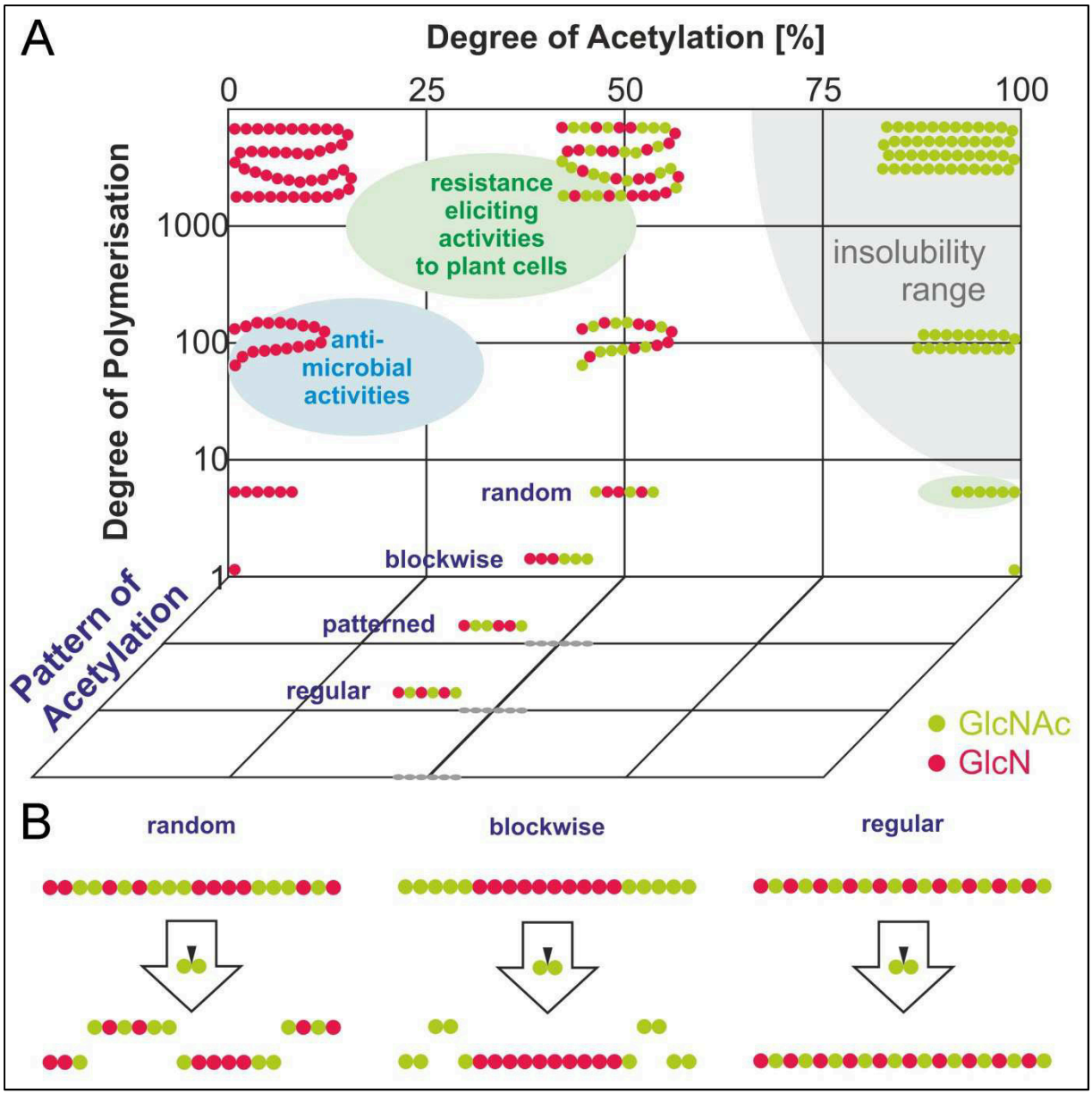

Fig. 1: Chitin and chitosan, A: Three-dimensional chitosan matrix, modified after El Gueddari et al. [2007], illustrating the properties degree of polymerization (DP), degree of acetylation (DA) and pattern of acetylation (PA) based on GlcNAc (green) and GlcN units (red). Chitosans assayed for antimicrobial [Oliveira et al., 2008] and plant eliciting activities [Vander et al., 1998] were produced chemically and possess a random PA [Weinhold et al., 2009]. **B: Cleavage of chitosan** with the same DP (20) and the same DP (50%), but different PAs (random, blockwise and regular) by a chitinase that hydrolyzes only between two GlcNAc units (green) result in different products or no cleavage in the case of a strictly regular PA

1.1.3. Pattern of acetylation

The sequence of acetylated and non-acetylated units it can be determined for oligomers by tandem mass spectrometry (MS^n) applying post source decay fragmentation comparable to peptide sequencing [Bahrke et al., 2002], even in complex mixtures [Haebel et al., 2007]. A new and simple method not requiring MS equipment is the enzymatic sequencing using N-acetylglucosaminidase, glucosaminidase and thin layer chromatography [Hamer et al., 2014]. While MS is widely used for the

analysis of chitosan oligomers including the PA determination, this method is not suitable for chitosan polymers without hydrolysis, which are usually investigated by nuclear magnetic resonance (NMR) measurements. ^{1}H-NMR is the validated and best method to determine DA by calculating the proportion of the peak representing the hydrogen of the acetate group to the peak areas of the hydrogen attached to the six carbon atoms in one unit [Hirai et al., 1991; Kasaai, 2010; Lavertu et al., 2003]. Using ^{13}C-NMR, the general randomness can be analyzed by comparing the relative intensities of the four possible diads AA, AD, DA and DD within the polymer describing the distribution of neighboring units [Kumirska et al., 2009; Vårum et al., 1991]. Although there has been controversy on chemical chitosan preparation [Aiba, 1991; Sannan et al., 1976; Varum et al., 1990], it has been shown that the PA of polymers is random, regardless which of the different preparation methods, homo- and heterologous deacetylation [Chang et al., 1997] or reacetylation [Trombotto et al., 2008], is applied. This has been confirmed by Weinhold et al. [2009], who also developed a formula to describe the pattern as value P_A between 0 and 2. Another possibility to analyze the PA is enzymatic/mass spectrometric fingerprinting with sequence-specific enzymes and comparative analysis of resulting products [Lopatin and Ilyin, 1995]. These analytical methods help to analyze enzymatic-modified chitosan with defined PA and comparing to chemically produced chitosans possessing random PA. Therefore, chitin deacetylases (CDAs) might be a valuable tool converting of chitin into chitosan, due to the regioselectivity of enzymatic reactions based on the subsite specificity and mode of action [Tsigos et al., 2000].

1.2. Chitin deacetylases

CDAs (EC 3.5.1.41) catalyze the hydrolytic cleavage reaction of the N-acetamido groups of GlcNAc residues in chitin and chitosan by releasing acetate, which classifies the enzymes into the carbohydrate esterase family 4 (CE4) of the Carbohydrate Active Enzymes database (CAZY, http://www.cazy.org) [Lombard et al., 2014]. Responsible for this reaction is the catalytic center with five conserved motives in the polysaccharide deacetylase domain (PDD), also named NodB homology domain due to the occurrence in the chitooligo-saccharide deacetylases of rhizobia [John et al., 1993], which is also present in peptidoglycan deacetylases and acetylxylan esterases [Caufrier et al., 2003]. The CDA enzyme was first described for the fungus *Mucor rouxii* in 1975 by Araki and Ito being active on glycol chitin, colloidal chitin and chitin oligomers DP 3-5 but not towards other substrates with GlcNAc residues. Later it was also found in bacteria, insects and several fungi; CDAs from fungi being

the most studied also in terms of biochemical properties, modes of action and biological roles as reviewed in Zhao et al. (2010).

1.2.1. Occurrence of CDAs and role in nature

For marine bacteria the role of chitin oligosaccharide deacetylases is part of the chitin catabolic pathway [Keyhani and Roseman, 1999] and the enzymes of some Vibrio species have been characterized, including *V. cholerae* [Li et al., 2007] and *V. alginolyticus* [Li et al., 2007]. As an example, *V. parahaemolyticus* degrades chitin using chitinase into dimers and then the chitin oligosaccharide deacetylase acts specifically on the reducing end [Kadokura et al., 2007]. The resulting product GlcNAc-GlcN functions as a signaling molecule for other deacetylating *Vibrio* bacteria by chemotactically sensing the presence of chitin [Hirano et al., 2011].

In contrast, CDAs of insects are not limited to a single role, because many different paralogous CDAs with the same localization are present in insects [Arakane et al., 2009]. Despite many studies on the function of putative CDA genes in gut physiology and development, the enzymatic activity of the CDAs has been confirmed only in some cases [Toprak et al., 2008; Zhong et al., 2014].

Like arthropods, fungi synthesize chitin as structural component and therefore these organisms express enzymes for synthesis, modification and degradation. In the case of zygomycetes chitosan is the mayor component of the cell wall [Bartnicki-Garcia, 1968] and believed to be synthesized by a tandem process of chitin synthase and chitin deacetylase as shown on *M. rouxii* [Davis and Bartnicki-Garcia, 1984]. A *M. rouxii* CDA was one of the first being purified and characterized [Kafetzopoulos et al., 1993] and its processive mode of action on polymers [Martinou et al., 1998] and oligomers [Tsigos et al., 1999] is explained by its role in nature. Also many other zygomycete CDAs, regarded as “intracellular type” based on their function, have been purified from culture supernatant or mycelium and investigated [Amorim et al., 2005], from species like *Absidia coerulea* [Gao et al., 1995], *Gongronella butleri* [Maw et al., 2002], *Mortierella* sp. DY-52 [Kim et al., 2008; Zhong et al., 2014], *Rhizopus circinans* [Gauthier et al., 2008] and *R. nigricans* [Jeraj et al., 2006].

In contrast to zygomycetes, yeast cell walls contain only small amounts of chitosan and CDAs are not necessary for vegetative growth, but for the cell walls of ascospores, as reported for *Saccharomyces cerevisiae* [Christodoulidou et al., 1999] and *Schizosaccharomyces pombe* [Matsuo et al., 2005]. Both

CDAs of *S. cerevisiae* have also been purified and characterized [Martinou et al., 2002; Mishra et al., 1997].

The first reported CDA activity from a non-zygomycete fungus was found in the plant pathogen *Colletotrichum lindemuthianum* [Kauss et al., 1983] and it has been proposed early, that this activity, also present in the pathogenic relative *C. lagenarium,* plays a role in host-pathogen interaction by escaping the plant chitinases and lowering the elicitor activity of emerging oligomers [Stegrist and Kauss, 1990]. This hypothesis was supported by a study that referred increased CDA activity of the rust *Uromyces viciae-fabae* during the development of infection structures [Deising and Siegrist, 1995]. A visual proof of the conversion of surface chitin into chitosan could be shown for the three species *Puccinia graminis* f. sp*. tritici, U. fabae* and *C. graminicola* by differential fluorescence staining of chitin and chitosan [El Gueddari et al., 2002]. This conversion seems to be important for the success of fungal infection, because resistant wheat was found to counteract fungal CDA activity [Maksimov et al., 2011]. Three CDAs expressed during vegetative growth of the opportunistic pathogen *Cryptococcus neoformans* may also function in a similar manner during the infection of humans, which have chitinolytic enzymes as well [Baker et al., 2007]. For the of CDA the entomopathogenic fungus *Metarhizium anisopliae* a dual role is suggested: the deacetylation of the own chitin and the weakening of the insect cuticle [Nahar et al., 2004].
On the other hand, the function of CDAs found in non-pathogenic ascomycetes like *Penicillium oxalicum* [Pareek et al., 2012] or *Aspergillus nidulans* [Alfonso et al., 1995] is not that clear, whereas for the CDA of the basidiomycete *Flammulina velutipes* could be linked to the fruiting body development [Yamada et al., 2006]. This CDA is also produced heterologously in *Pichia pastoris* for characterization studies [Yamada et al., 2008].

1.2.2. Characterization of CDAs

Many CDAs have been isolated directly from the fungus itself or have been heterologously expressed in *P. pastoris* or *Escherichia coli* including. Examples are the *A. nidulans* CDA in the *E. coli* pET vector system [Wang et al., 2009] and the most studied *C. lindemuthianum* CDA in both expression systems [Shrestha et al., 2004; Tokuyasu et al., 1999]. In case of the *M. rouxii* CDA, the processive mode of action on oligomers and polymers could be solved [Martinou et al., 1998; Tsigos et al., 1999], whereas the deacetylation mode of *C. lindemuthianum* CDA differs clearly from the zygomycete CDA by

deacetylating single GlcNAc units more randomly [Tsigos et al., 2000]. Detailed analysis of oligomer deacetylation led to the model of four subsites with different affinities [Hekmat et al., 2003; Tokuyasu et al., 1997; Tokuyasu et al., 2000a]. This finding could be supported by the crystal structure, which was the first for a CDA, revealed insights into the enzyme mechanism and confirmed the role of the Zn^{2+} cofactor [Blair et al., 2006].

Up to now, investigated CDAs vary naturally in a certain range of optimal conditions for activity like pH, temperature and presence of divalent cations, as well as in their molecular weights due to N-glycosylation [Zhao et al., 2010]. It should be stated, that the values in literature are often difficult to compare, because of the variety of methods and substrates for CDA activity measurements. The most common is the estimation of acetate release, either enzymatically by three coupled reactions [Bergmeyer, 1974; Martinou et al., 1995] or by gas chromatography [Aye et al., 2006] and the use of substrates with radiolabeled N-acetyl groups is still applied [Araki and Ito, 1975]. On the other hand the quantification of free amino groups is possible with fluorescamine [Blair et al., 2005] and zymogramms can also be generated by using glycol chitin in polyacrylamide gels and Calcofluor white staining [Trudel and Asselin, 1990]. Besides the partly O-hydroxyethylated derivative glycol chitin other soluble substrates are chitin oligomers DP 2-6 and chitosans with DA 30-60%. The CDA activity on crystalline substrates like α- and β-chitin is commonly low and can be enhanced by treating the material for reduced crystallinity, like colloidal chitin [Beaney et al., 2007] or “superfine” chitin, and thereby for a better accessibility of the acetyl groups [Win and Stevens, 2001]. In contrast to chitinases, CDAs seem to be only capable of acting on the surface of chitin particles [Aye et al., 2006]. Hydrolytic enzymes of insoluble substrates like cellulases and chitinases often contain additional domains for carbohydrate-binding and improved hydrolysis. Interestingly, there are CDA genes available with this modular structure in databases and described for some insect species [Dixit et al., 2008; Wang et al., 2006] as well as for the fungus *Magnaporthe grisea* [Kamakura et al., 2002], however, the activity of these CDA hasn’t been explored, yet.

1.3. Carbohydrate-binding domains

In general, carbohydrate-binding modules (CBMs) represent certain protein folds with a binding activity towards sugar molecules. Due to the fact that folds are more conserved than amino acid sequences, CBMs are classified in families according to their fold in the CAZY database [Lombard et al., 2014], since they are often associated with carbohydrate active enzymes. These non-catalytic domains are also listed in the Pfam database, which is based on hidden Markov model of multiple sequence alignments [Finn et al., 2014]. After three-dimensional structures of different folds have been solved, the CBM families were sorted in three types by the position of the substrate-binding aromatic residues for stacking with the sugar rings by van der Waals interactions. CBMs of type A build a planar layer of aromatic rings for the interaction with crystalline surfaces. Modules of type B form an extended groove that targets several sugar units on a single polysaccharide chain and type C is characterized by binding small sugar molecules in a more pocket-like structure and forming hydrogen bond interactions as well [Boraston et al., 2004; Hashimoto, 2006].

These modules are mainly found in carbohydrate-active enzymes that are multidomain proteins and occur solely or in tandem in binding proteins such as lectins. The role of CBMs in hydrolytic enzymes like cellulases and chitinases is to recognize and bind the crystalline substrates and thereby concentrate these effectively on the surface for an enhanced activity [Guillén et al., 2010]. In some cases it was reported, that CBMs are able to disrupt the substrate without hydrolysis [Shoseyov et al., 2006], e.g. on cellulose fibers [Din et al., 1991]. For another cellulose-binding module it has then been shown to change the cellulose fibril structure by weakening the hydrogen bond network [Wang et al., 2008a]. Another example is the chitin-binding protein CBP21 from chitinolytic *Serratia marcesens,* which helps the bacterium's chitinases by loosening the surface and increasing the substrate accessibility [Vaaje-Kolstad et al., 2005].

1.3.1. Chitin binding domains

Within the number of CBMs described until now, there are 12 families with chitin-binding activity demonstrated at least in one case. These chitin binding domains (CBDs) occur in lectins common to plants [Damme et al., 1998], chitin recognition proteins, which are often associated with the Lysin Motif [Buist et al., 2008] and in chitinases. The binding properties of CBDs in bacterial [Blaak and Schrempf, 1995; Hashimoto et al., 2000] Huang et al., 2006] and insect chitinases [Arakane et al., 2009] have

been investigated by analysis of truncated enzymes, demonstrating that CDBs are not absolutely required for hydrolysis, but strongly improve the binding of insoluble chitin.

Amongst plant [Beintema, 1994] and fungal chitinases [Hartl et al., 2012] the hevein-like CBD of CBM family 18 is the most prevalent, occurring only in eukaryotes and also referred as 'chitin binding 1', as seen in Pfam. The structure of CBM18, which has its own Hevein fold family, is known from wheat germ agglutinin (WGA) [Wright et al., 1991]. To date only one crystal structure of CBM18 within a chitinase is available and yet the proline- and threonine-rich linker region is predicted to be flexible [Kezuka et al., 2010]. The domain itself is about 40 amino acids small and the three-dimensional structure is stabilized by four disulfide bonds formed by the characteristic cysteine residues [Beintema, 1994].

After it had been demonstrated that a CBM18 of *Nicotiana tabacum* chitinase has chitin binding activity and is responsible for high affinity to the crystalline substrate [Iseli et al., 1993], in 2001 Limón and colleagues showed that the addition of the plant CBD to a fungal chitinase resulted in higher hydrolytic activity towards insoluble chitins. Other chimeric chitinases showed similar results [Huang et al., 2009; Matroodi et al., 2013; Neeraja et al., 2010]. CBDs of chitinases are becoming widely studied, however, their biochemical role in CDAs has not yet been investigated, although the presence of these sequences is known [Dixit et al., 2008; Luschnig et al., 2006]. Assuming a similar role for CDA like for chitinases, CBDs could enhance the deacetylation activity on crystalline chitin substrates. Databases contain a number of fungal CDA sequences inclosing one or more family 18 CBDs, which were to my knowledge not topic of any further research and therefore a good source for novel enzymes to be characterized biochemically.

1.4. Aim of the thesis

Topic of the present doctoral thesis is the study of chitin deacetylases that feature one or more chitin binding domains in a multi-domain structure. Investigating these special types of enzymes provides an opportunity to discover novel CDAs with unknown qualities, regarding substrate specificity, mode of action and produced pattern of acetylation.

The aim of this research was to identify, produce and characterize new enzymes by a knowledge based approach, meaning to start with putative CDA sequences listed in databases. After bioinformatic analysis on domain structures and sequence similarities to known CDAs, candidates should be cloned with affinity tags for heterologous production in three host systems available (*Escherichia coli, Schizosaccharomyces pombe* and *Hansenula polymorpha*), in order to obtain correctly folded and active enzymes for characterization. One putative CDA gene from *Botrytis cinerea* (BC95) containing three N-terminal CBDs and one from *Podospora anserina* (PaCDA) with one N-terminal and one C-terminal CBDs were chosen. The biochemical characterization of CDAs includes tests on optimal temperature and pH, influence of bivalent cations and possible inhibitors, assays for substrate specificity and demonstrating the mode of action. Furthermore, truncation variants of PaCDA were created lacking the N-terminal (ΔN), the C-terminal (ΔC) or both domains (ΔNC), to investigate the role of CBDs for PaCDA and their binding activity towards various substrates like it is reported for chitinases.

Besides testing the enzymatic properties, a second focus was set on the CDA products, to generate chitosans with non-random pattern of acetylation. This will lead to further insight into the influence of PA on physicochemical properties and biological activities of chitosans. The CDA product, which cannot be obtained by chemical methods, might have different biological activities as known chitosans, which are tested comparatively to chemically produced chitosan possessing the same DP and DA. If differences can be demonstrated, enzymatically produced chitosans with non-random PAs represent a promising starting-point for novel applications.

Chapter 2

Expression during conidia germination and heterologous production of *Botrytis cinerea* putative chitin deacetylases

Abstract

Chitin deacetylases (CDAs), enzymes converting chitin to chitosan, are known from several fungi, were they take part in cell wall formation or plant-pathogen interaction. The genome of *Botrytis cinerea* contains six putative CDA sequences; of these three possess additional chitin binding domains (CBDs). I studied the expression pattern of these genes during conidia germination, the main inoculum of this plant pathogen. BC1G_00895 (BC95) was confirmed to be upregulated 4 h after germinating in fructose containing Gambourg's B5 medium. The gene was chosen for heterologous protein production, because of its uncommon domain architecture containing three N-terminal CBDs. Due to these CBDs, the protein includes many disulfide bonds. Hence, the protein production was expected to be challenging. Therefore, different expression systems for protein production including the use of the hosts *Schizosaccharomyces pombe, Escherichia coli,* and *Hansenula polymorpha* were tested. Production of BC95 was indeed successful in *H. polymorpha*, but the protein remained intracellular despite the secretion signal of the proteins signal peptide. CDA activity on the substrates glycol chitin, colloidal chitin, chitosan (DA 56%) and chitin oligomer still remains to be proven.

2.1. Introduction

Chitosan, the linear heteropolymer of β-1,4-linked glucosamine (GlcN) and N-acetylglucosamine (GlcNAc) units, has become of increased interest due to its wide range of application possibilities such as in medicine [Hu et al., 2013; Jayakumar et al., 2011], plant protection [El Hadrami et al., 2010] and water treatment [Bhatnagar and Sillanpää, 2009]. Commercially it is mainly made from chitin-containing shrimp shells, which turns the shrimp residues into a profitable product. The term chitosan refers to a diverse group of chitinous molecules differing in size and number of acetyl groups. These properties are defined as degree of polymerization (DP) and degree of acetylation (DA), respectively. Studies have shown that these characteristics of chitosan are influencing its applicability due to differences in physicochemical properties [Aranaz et al., 2009], but the structure-function-relationships are so far not completely understood. For this purpose the pattern of acetylation (PA) is considered as an important third parameter, especially for the biological activities of chitosan, due to sequence specific hydrolases like plant chitinases [Brunner et al., 1998] and human chitotriosidase [Renkema and Boot, 1995] present in the target tissues.

Chitosan was first discovered by Kreger in 1954 in a zygomycete fungus and it was reported to be a major cell wall component and the characteristic polymer for this phylum [Bartnicki-Garcia, 1968]. Later it was also found in other fungal phyla (basidio- and ascomycota). Also, genes for its enzymatic conversion named chitin deacetylases have been identified [Tsigos et al., 2000].

Chitin deacetylases (CDAs) convert N-acetylglucosamine within the polymer to glucosamine units by hydrolysis of the acetamido group and the release of acetate (EC 3.5.1.41). Although some bacterial enzymes have been identified by now, fungal CDAs are more widely studied and enzymatically characterized [reviewed in Zhao et al. 2010).

Amongst others, CDA genes from *Aspergillus nidulans* [Wang et al., 2008b] and *Rhizopus circinans* [Gauthier et al., 2008] have successfully been heterologously expressed in *Escherichia coli* and *Pichia pastoris*, respectively; the most investigated *Colletotrichum lindemuthianum* CDA [Shrestha et al., 2004; Tokuyasu et al., 1999] was expressed even in both systems. Besides *C. lindemuthianum*, other plant pathogenic species were also found to possess active CDAs. The presence of active CDAs has been confirmed e.g. in *Aspergillus nidulans* [Alfonso et al., 1995], *Penicillium oxalicum* [Pareek et al., 2011] and *Colletotrichum gloeosporioides* [Pacheco et al., 2013]. In the fungi *Colletotrichum lagenarium* [Stegrist and Kauss, 1990], *Uromyces viciae*-fabae [Deising and Siegrist, 1995] and

Puccinia graminis f. sp. tritici [El Gueddari et al., 2002] CDA activity has been proposed to play an important role during plant infection.

Botrytis cinerea is a plant pathogen attacking more than 200 host species and a necrotrophic life style which leads to the so called grey mold disease [reviewed by van Kan 2006]. Conidia are the main inoculum for grey mold disease. They germinate with a short germ tube and develop then an appressorium to penetrate the plant epidermal [Williamson and Tudzynski, 2007]. Genes expressed in this state of development encode enzymes such as cutinase [van Kan et al., 1997], lipase [Comménil et al., 1995] and several plant cell wall degrading enzymes. Relating to virulence, delayed lesion formation was observed after deletion of pectinases (endo-polygalacturonases) *Bcpg*1 [ten Have et al., 1998] and *Bcpg*2 [Kars et al., 2005] or β-1,4-xylanase [Brito et al., 2006]. A putative CDA gene is strongly induced in the first hours of germination and therefore described as germination-specific marker [Leroch et al., 2013], but the enzymatic function has never been investigated. This sequence, as well as few more putative CDAs in *B. cinerea*, contains additional carbohydrate binding modules [Amselem et al., 2011].

Carbohydrate binding modules (CBMs) are mainly found in enzymes that degrade insoluble polymers like cellulases but also occurr individually in binding proteins and lectins [Boraston et al., 2004]. To date, a large number of CBMs with different functions are described and classified into families in the CAZY database (http//:www.cazy.org) [Lombard et al., 2014]. CMB18, functionally characterized as type 1 chitin binding domain (CBD, PFAM 00187), is present in eukaryotes only and it's HMM (Hidden Markov Model) pattern contains eight characteristic cysteine residues [Wright et al., 1991]. Analysis of the three-dimensional structure confirmed the presence of four disulfide bridges and a unique fold named hevein, from *Hevea brasiliensis* [Beintema, 1994]. Genome databases contain numerous uncharacterized sequences with CBDs along with other domains including polysaccharide deacetylase domains (PDD), but CDA activity of encoded proteins was not investigated so far. Until now the direct enzymatic conversion of chitin into chitosan is only possible to a small extent because the insoluble chitin is a poor substrate for all known CDAs [Zhao et al., 2010]. The presence of one or more CBDs could enhance this activity, as it is known for chitinases containing CDBs [Blaak and Schrempf, 1995; Hashimoto et al., 2000; Iseli et al., 1993].

The aim of this study is to identify, produce and characterize a novel chitin deacetylase with chitin binding domains and possible new properties, substrate preferences or modes of action. As the production of one putative CDA from *Botrytis cinerea* (BC95) emerged to be challenging, three different pro- and eukaryotic expression systems were investigated: *Schizosaccharomyces pombe* (fission yeast), capable of glycosylation and forming disulfide bridges; an *E. coli* strain improved for rare codon usage and reducing environment, and *Hansenula polymorpha,* a Pichia strain engineered for recombinant protein production.

2.2. Materials and methods

2.2.1. Bioinformatics

A search for putative chitin deacetylases in *Botrytis cinerea* was carried out using the GenBank (http://www.ncbi.nlm.nih.gov) in March 2009. The Pfam database (http://pfam.sanger.ac.uk) was accessed to identify domains present and show their architecture. Signal peptides were identified by SignalP 3.0 (http://www.cbs.dtu.dk/services/ SignalP) [Bendtsen et al., 2004] and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) was used to predict potential N-glycosylation sites [Blom et al., 2004]. Multiple sequence alignments and cladograms were computed by ClustalW2 (http://www.ebi.ac.uk/Tools/ clustalw2) [Larkin et al., 2007].

2.2.2. Cultivation of *Botrytis cinerea*

Botrytis cinerea B05.10 was kindly provided by P. Tudzynski (University of Münster, Germany) and grown on potato dextrose agar at room temperature and light conditions (16 h light with UV, 8 h dark) until sporulation occurred (10-12 days). The conidia were harvested by adding 10 ml sterile water and scraping with a glass spatula, washed three times and quantified [Doehlemann et al., 2006].

For germination $5 * 10^6$ conidia per ml were incubated in 14.5 cm diameter glass petri dishes with 50 ml bi-distilled water or Gambourg's B5 medium (Duchefa, Haarlem, The Netherlands) containing 10 mM fructose at 20°C. Germinated conidia were harvested at different time points by scraping off the glass and separating from the germination medium by centrifugation (5000 g, 4°C, 10 min). Medium was concentrated for activity assays using Vivaspin 20 ultrafiltration devices with 10 kDa molecular weight cut-off (Sartorius AG, Göttingen, Germany) and mycelium was washed by centrifugation (5000 g, 4°C, 5 min) and frozen at -80°C.

2.2.3. DNA/ RNA isolation and RT-PCR

Botrytis cinerea DNA, isolated from mycelium using a protocol with lysis at 60°C and isopropanol extraction, was kindly provided by Dr. Carolin Richter. RNA isolation from frozen samples of germinated conidia incubated for 4 h was carried out by following the protocol described by Richter et al. [2012]. Obtained RNA pellets were dissolved in 50 µl water (RNase-free) and RQ1-RNAse-Free DNAseI was used according to the manufacturer's instructions (Promega, Mannheim, Germany) to remove DNA contaminants. Two extraction steps were subsequently carried out as described earlier [Richter et al. 2012]. RNA samples were dissolved in 30 µl RNase-free water and spectrophotometrically quantified.

The cDNA synthesis was carried out using 1 µg of RNA samples, M-MLV reverse transcriptase (Promega) and Oligo (dT18) primers, following the manufacturer's recommendations. Gene-specific primers applied for PCR amplification with Mango*Taq*™ DNA polymerase (Bioline, London, UK) are listed in table 1. Separation of the fragments was carried out by electrophoresis with 1% agarose gels and visualization by ethidium bromide staining and UV light.

Table 1: Oligonucleotide primers for amplification of *B. cinerea* putative CDA sequences (GenBank) and actin control, length of obtained fragments given for RNA and DNA and sequences from 5' to 3'.

gene/primer name	fragment length (RNA/DNA) bp	sequence 5' => 3'
BC1G_03291	323 / 523	GTTGCTGTGGCCGGAAGC CTGGGCGGGCAATGGTAG
BC1G_06509	321 / 370	GGCTTCGTTGTCAAGTGCAC GTAGAACCACCAGCTGGAAC
BC1G_00895	730 / 781	CCAACCACAGGAGGAACTTG GAACGGCTGTTGAAGCAAGC
BC1G_09146	488 / 537	CCCTTGGCCAATGGACAAGTG GCTACTTGTCACAACAGCCCC
BC1G_08249	694	CCTGGTGCTCCTAGCTCTAC GCCTCCTAGCGATTCAACCC
BC1G_14964	445 / 556	GAGACTCGGAGATACTTGGAC CTTCCTTAGGACTTCTCCAGC
BC actin	250 / 267	AAGTGTGATGTTGATGTCC CTGTTGGAAAGTAGACAAAG

2.2.4. CDA activity tests

CDA activity was tested in germinated conidia and mycelium after a germination period of 2.5, 8 and 24 hours. For each condition the medium was concentrated and rebuffered to phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) to collect the extracellular protein fractions, which were tested along with cell extracts, cell wall extracts and mycelium pellets on activity gels. Therefore the material was grinded in liquid nitrogen before addition

of lysis buffer (20 mM Tris/HCl pH 8.0, 0.05% Triton-X 100, 150 mM NaCl and 2% (v/v) protease inhibitor cocktail for tissue culture, Sigma-Aldrich, St. Louis, USA), mixed at 4°C for 15 min and centrifuged at 8500 g and 4°C for 5 min. Remaining pellets were washed with 1M NaCl to remove ionic bound proteins (cell wall extracts) and pellets afterwards were tested as mycelium residue samples. Purified recombinant CDA from *Puccinia graminis* sp. f. *tritici* or *Podospora anserina* were used as positive controls for all CDA activity tests.

CDA activity gels were prepared as previously described by Trudel & Asselin [1989] with 0.1% (w/v) glycol chitin, 12% (w/v) polyacrylamide, 12.5 mM buffers (BisTris pH 6.0 and 7.0, borate pH 8.0) and 1 mM $ZnCl_2$. Samples were applied onto the gels (dot gels) as drops of 3 µl each or equal sized solid mycelium pieces. Gels were incubated overnight at 37°C, followed by staining with Calcofluor White (Fluorescent Brightener) and chemical depolymerization of deacetylated chitin [Trudel and Asselin, 1990].

2.2.5. Heterologous production of BC95

2.2.5.1. Nucleic acid manipulations: Cloning of BC95

The sequence of putative CDA BC1G_00895 (BC95) was amplified via PCR using the cDNA sample from 4 h germinated conidia in fructose medium and primers BC95_forward: 5'-AGACCATGGTTTTC CAGTGGAGGGAACTGATTC-3' including *Nco*I restriction site and BC95_reverse: 5'-CTCGTCGACT TAATGATGATGGTGATGATGATAACCAAAAGCGGTATTCG-3' introducing a C-terminal His_6-Tag (underlined) and *Sal*I restriction site. The product of proof-reading *Pfu*-polymerase had a size of 1825 bp confirmed by electrophoresis and was extracted from the gel. After polyadenylation, the BC95 gene was inserted to pCR®II-TOPO® cloning vector using the TOPO cloning kit according to the manual (Invitrogen/Life Technologies) before transformation of *Escherichia coli* with the pCR®II-TOPO::BC95-His_6 plasmid. This plasmid was used for cloning the BC95 gene for further experiments into expression vectors.

2.2.5.2. Biochemical analytics

Protein samples obtained from three different expression hosts specified below were analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) as described [Laemmli, 1970],

using gels containing 12% (w/v) polyacrylamide. After electrophoresis gels were either used for silver staining [Yan et al., 2000] or for western blotting onto a nitrocellulose membrane in semi-dry method. Subsequently reversible PoncausS staining (solution 0.1 % (w/v) in 5% acetic acid, Sigma-Aldrich) was carried out before immunodetection with His•Tag® Monoclonal Antibody (mouse-IgG, Novagen, Merck KGaA, Darmstadt, Germany) and goat-anti-mouse-IgG-HRP-conjugate, (horseradish peroxidase, Sigma-Aldrich) or Strep-Tactin® HRP conjugate (IBA, Göttingen, Germany) followed by enhanced chemi-luminescence (ECL) detection. Molecular weight markers used during protein separation were Precision Plus^{TM} Protein All Blue Standards (Bio-Rad Laboratories GmbH, München, Germany) and unstained, StrepII-tagged peqGOLD Protein-Marker II (Peqlab Biotechnologie GmbH, Erlangen, Germany).

De-N-glycosilation of protein samples was carried out by using PNGase F (New England Biolabs, Ipswich, USA) following the manufacturer's protocol. For protein quantification, either the bicinchoninic acid assay (Pierce^{TM} BCA Protein Assay Kit, Thermo Fisher Scientific Inc., Waltham, USA) or the Bradford assay [Bradford, 1976] was used.

To detect CDA activity, glycol-chitin gels were prepared as described above (details stated below in section for each case) and samples incubated with different substrates were analyzed for acetate release by threefold coupled enzyme assay [Martinou et al., 1995]. Therefore an acetic acid assay kit was used according to the manufacturer's recommendations (R-Biopharm AG, Darmstadt, Germany), but downscaled for 96-well mircotiterplates, and acetate concentrations were calculated against a set of standards.

2.2.5.3. *S. pombe* – cloning

For the construction of expression strains, the fission yeast *Schizosaccharomyces pombe* and the pMel vector with *Saccharomyces cerevisiae* melibiase signal peptide for extracellular production were chosen. The vector is a pREP derivate. It carries a thiamin-repressible *nmt1*-promoter, a *nmt1*-terminator sequence [Maundrell, 1993] and the *ura4*-gene for selection in uracil-auxotrophic strains [Grimm et al., 1988]. The BC95 gene was cut out from the pCR®II-TOPO::BC95-His_6 plasmid using *Nco*I and *Sal*I restriction enzymes, extracted from an agarose gel and ligated to the pMel, which was also digested by these two enzymes before. *S. pombe* ura4-D18 was cultivated on YES agar with 225 mg/l uracil and transformed following the short protocol described by Forsburg & Rhind [2006].

In further experiments, the His_6-tag was exchanged to a StrepII-Tag [Schmidt and Skerra, 2007] coding sequence by amplifying the whole plasmid with back-to back phosphorylated primers: 5'-pho-CAATTTGAAAAGTAACATCATCACCATCATCATTAAGTCG-3' and 5'-pho-AGGATGAGACCAATAAC CAAAAGCGGTATTCGAAC-3', coding for one half of the tag each (StrepII sequence underlined) and Phusion™ Hot-Start High-Fidelity DNA Polymerase (Finnzymes, Thermo Scientific). The PCR template pMel::BC95-His_6 was then digested using restriction enzyme *Dpn*I followed by recirculation of the plasmid pMel::BC95-StrepII.

2.2.5.4. *S. pombe* – screening

Transformants of *S. pombe* were cultivated on Edinburgh minimal medium (EMM) or EMM agar (20 g/l Difco™ Agar, BD, Sparks, USA) including adenine, histidine, leucine and lysine-hydrochloride (225 mg/l each), but without uracil to maintain the pMel plasmid. To screen *S. pombe* clones, 2-ml pre-cultures with 10.1 g/l thiamine were incubated at 30°C and 200 rpm for 30 h followed by main cultures containing 20 ml EMM without thiamine to induce gene expression by the *nmt1*-promoter. After 30 h incubation at 30°C and 200 rpm, culture supernatant was harvested by centrifugation (1500 g, 4°C, 5 min) at OD_{600} 8-10, rebuffered in PBS and concentrated 200-fold using Vivaspin 20 ultrafiltration devices with 10 kDa molecular weight cut-off (Sartorius AG). Cell extract samples were prepared following the small-scale protein preparation for fission yeast [Forsburg and Rhind, 2006]. SDS-PAGE and western blotting were carried out as describe above. Enzyme activity of 3-µl samples was tested on glycol chitin gels at different pH values with buffers sodium citrate (pH 5.0), BisTris (pH 6.0, 6.9 and 7.0) and borate (pH 8.0 and 9.0).

2.2.5.5. *E. coli* – cloning

For heterologous production of the putative CDA BC95 in *E. coli*, the Novagen vector pET-22b(+) (Merck) was used. The BC95 coding sequence was amplified via PCR from pMel::BC95-His_6 while His_6-Tag coding sequence was replaced by the StrepII-Tag and simultaneously restriction sites (*Fau*I + *Xho*I) were introduced with the primers 5'-CCCGCAATTTATGAAGCTTCGGATCCC TTTTCCAGTGGAGGGAACTG-3' and 5'-CCGCTCGAGCTATTTTTCAAATTGAGGATGTGACCAATA ACCAAAAGCGGTATTCGAAC-3' (StrepII-Tag coding sequence underlined). For the thioredoxin A coding sequence, a colony PCR on *E. coli* K12 was carried out using primers TRX_for (5'-CCCAAGCTTAGCGATAAAATTATTCACCTGAC-3)' and TRX_rev (5'-CGGGATCCTCGCCAGGT

TAGCGTC-3') with *Hind*III and *Bam*HI restriction sites, respectively. These sites were used to clone it in front of the BC95-StrepII coding sequence in order to get pET-22b(+)::TrxA-BC95-StrepII expression plasmid. Expression strain for all constructs was *E. coli* Rosetta 2 (DE3) [pLysS-RARE2] carrying a plasmid for *E. coli* rare codons with chloramphenicol resistance cassette.

2.2.5.6. *E. coli* – screening

Screening for protein production was carried out using the T7 expression systems in auto induction medium as described by Studier [2005], adding 0.1 mM $ZnCl_2$, 125 µg/ml ampicillin and 34 µl/ml chloramphenicol. In the first experiment baffled flasks with the addition of 20 mM $MgSO_4$ were used additionally. In a second experiment non-induced cultures were used as controls and grown in LB medium (Luria/Miller, Roth) without induction supplements. Expression strains were incubated at 37°C and 180 rpm for 2 h, then at 26°C and 120 rpm for 16 h. After freezing over night at -20°C, cells were resuspended in PBS and 0.5 µl Benzonase® nuclease (Novagen, Merck) was added to the auto-lysed cell samples. Cell pellets were separated from cell extracts by centrifugation at 15,700 g and 4°C for 1 min and both protein fractions (1 µl each) were tested on CDA activity gels with 12.5 mM BisTris buffer pH 6.9 at 37°C and 20°C.

2.2.5.7. *H. polymorpha* – cloning

The protein sequence of BC95 including the StrepII-Tag was provided to GeneArt® (Thermo Scientific) for codon-optimization and synthesis for heterologous production in the uracil-auxotrophic (ura3) *H. polymorpha* host strain RB11 [Zurek et al., 1996]. The synthetic gene was then used by Artes Biotechnology GmbH (Langenfeld, Germany), to construct expression plasmids by cloning it into pFPMT121 [Degelmann et al., 2002] with the *FMD* (formate dehydrogenase) promoter, the *MOX* (methanol oxidase) terminator and an upstream extension of the pre-propeptide MF-α1 coding sequence of *S. cerevisiae* for secretion [Kurjan and Herskowitz, 1982]. After preparation of competent cells and transformation [Klabunde et al., 2007], uracil-prototrophic transformants underwent repeated cycles of growth under selective and nonselective conditions (passaging and stabilization), to promote mitotically stable integration of multiple expression plasmid copies into the yeast genome.

2.2.5.8. *H. polymorpha* – screening

Pools of stabilized transformants were pre-screened by Artes Biotechnology by cultivation in glycerol-containing medium to induce expression (YPYNBG, 1% yeast extract, 2% peptone, 0.14% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulphate, 2% glycerol, 0.1 M potassium phosphate buffer pH 6.0) at 37°C for 70 h. In other experiments varying temperatures and media compositions were tested and different cell fractions were analyzed by western blots with Strep-Tactin-HRP conjugate (IBA) and chromogenic detection.

I received the so-called pools #1, #4 and control strain "Mock" from Artes and screened cell-free culture supernatants as well as cell extracts for CDA activity on glycol chitin gels as described above with 2 µl samples and 25 mM phosphate borate buffer pH 8.0. CDA activity tests for the substrates colloidal chitin (1 g/l), chitosan DA 56% (0.5 g/l) and chitin pentamer (0.25 g/l) were carried out by incubation of 10 µl-samples in 210 µl 50 mM phosphate borate buffer pH 7.0 at 37°C and 750 rpm for 43 h and measuring acetate release as described above.

Cell extracts were prepared by resuspending cell pellets in pre-cooled (4°C) disruption buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) with appropriate volumes in order to achieve an OD_{600} of 60. Disruption was carried out with 500 µl acid-washed glass beads (Ø 0.5 mm) for 500 µl cell suspension samples and agitation on a Vibrax® (IKA, Staufen, Germany) at maximum speed and 4°C for 10 min. Thereafter 500 µl disruption buffer was added for another 10 min of agitation and extracts were cleared by centrifugation (500 g, 4°C, 2 min).

2.3. Results

2.3.1. Bioinformatical analysis

The search for domain architectures combining polysaccharide deacetylase (PDD) and chitin binding domain (type CBM 18) yielded six sequences of *Botrytis cinerea* putative CDAs, all differing in length and domain architecture (Fig. 1). Four of the obtained sequences contained a signal peptide and three shared additional homologies to chitin binding domains (CBDs). Those three sequences containing CBDs clustered in one group of the cladogram, whereas the remaining sequences built a second group. Alignments of the six PDDs only revealed also that the three sequences including CBDs are more similar to each other than to any of the other sequences from *B. cinerea* CDAs.

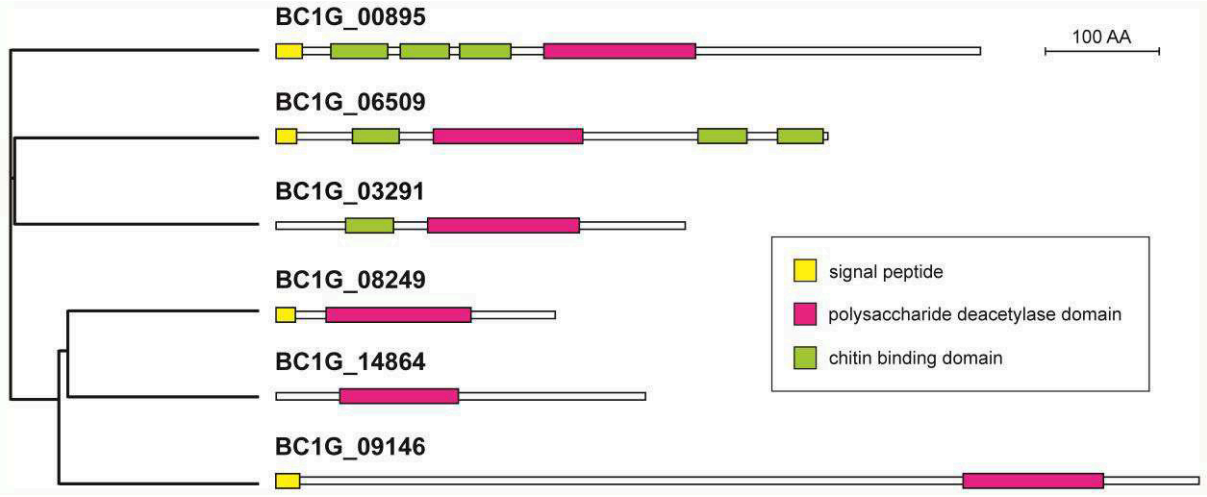

Fig 1: Cladogram and schematic structure of *Botrytis cinerea* putative chitin deacetylases (CDAs): sequences accessed from GenBank, multiple sequence alignment by ClustalW2, yellow: signal peptide (identified by SignalP 3.0), pink: polysaccharide deacetylases domain (PF01522) and green: chitin binding domain (CBD, PF00187) identified by Pfam. CDAs without CBDs cluster in one group separated from the CDAs containing one or more CBDs.

BC1G_00895 (BC95) has three N-terminal chitin binding domains, which differ not only in their sequences but also in their length. The genomic 1978-nucleotide-sequence has two introns and encodes for a protein composed of 618 amino acids and 64.8 kDa in size: this includes a 23 amino acid signal peptide. In addition, the secreted enzyme has six possible N-glycosylation sites, of which three show the utmost probability.

2.3.2. Gene expression during conidia germination

To evaluate if the identified sequences belong to expressed genes, an expression study using germinated conidia was carried out (Fig. 2). The experiment revealed bands for all six CDA sequences, however, the obtained band for BC1G_09146 was weak and was absent using fructose medium instead of distilled water. In contrast, the gene for BC95 seemed to be stronger expressed in the medium containing fructose, whereas the remaining four putative CDAs were expressed at the same level under all conditions tested. Germination and growth of the mycelium was much slower in bi-distilled water compared to the fructose medium, as seen by visual inspection.

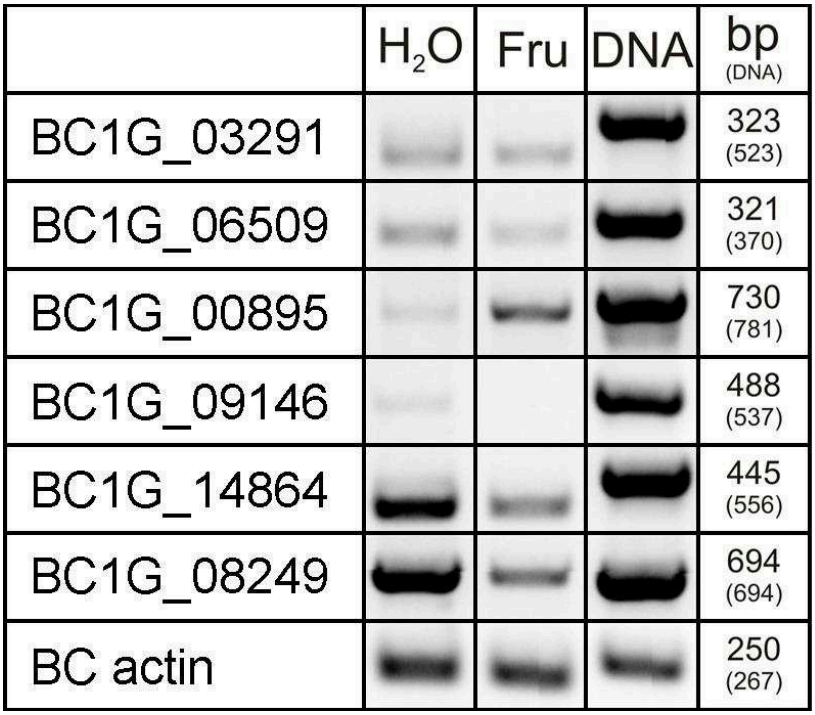

Fig. 2: Reverse transcription-PCR analysis of putative CDA gene expression during *Botrytis cinerea* conidia germination: RNA samples collected 4 h after inoculation in different media, H_2O: bi-distilled water, Fru: Gambourg's B5 medium with 10 mM fructose, controls: genomic DNA as template and primers for *B. cinerea* actin, fragment sizes given for RNA and DNA (bp). BC1G_00895 (BC95) shows enhanced expression in fructose containing medium in comparison to H_2O.

2.3.3. CDA activity during conidia germination

Because all putative CDAs were expressed during germination, it was tested if any CDA activity was present. Different fractions were used for this experiment: concentrated growth medium, crude cell extracts, cell wall extracts and pelleted mycelium, all at 2.5, 8 and 24 h after germination. Culture supernatant did not show any activity, even after concentration (data not shown). Chitinase activity was detected using a dot assay containing glycol chitin at pH 6.0 that was most intense for the

pelleted mycelium samples and present as well in the cell extract 24 h after conidia inoculation (Fig. 3). For all other samples neither CDA nor chitinase activity was observed using dot assays at pH 7.0 and 8.0 (data not shown).

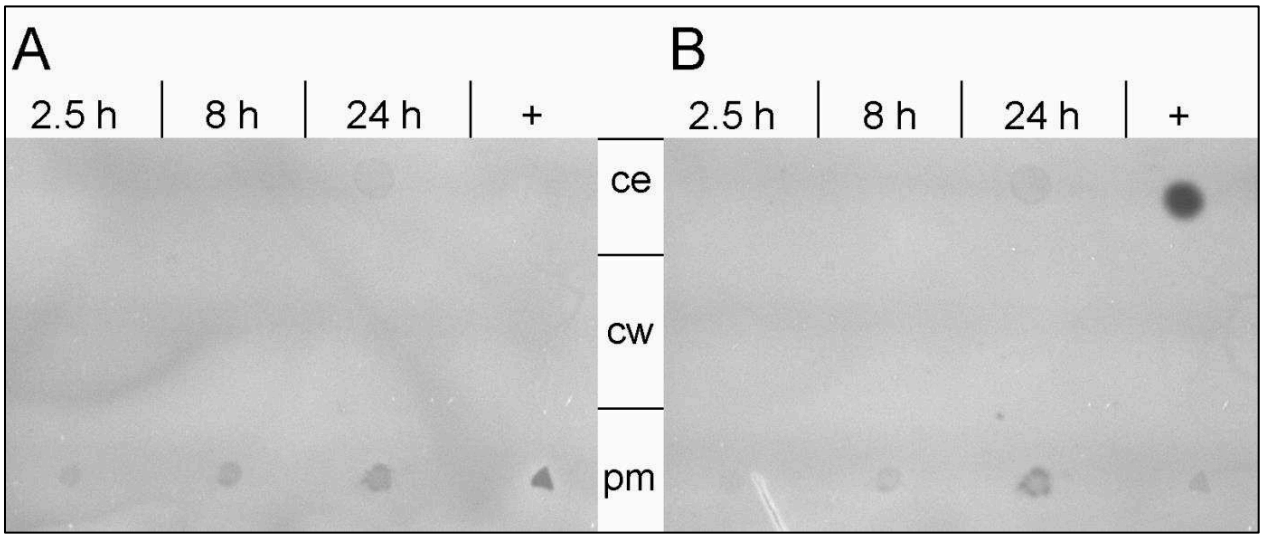

Fig. 3: Chitin deacetylase activity test using 0.1% glycol chitin at pH 6.0, before (A) and after depolymerization of chitosan (B). Samples of germinated conidia collected 2.5, 8 and 24 h after inoculation in Gambourg's B5 medium with 10 mM fructose, **ce**: cell extract, **cw**: cell wall extract, **pm**: pelleted mycelium, stained with Calcofluor white. Dark spots present only after depolymerization represent CDA activity (B), spots present before (A) are due to chitinolytic activity.

2.3.4. Heterologous production of BC95

Botrytis cinerea putative chitin deacetylase BC95 was chosen for heterologous production because of its highly interesting architecture including three chitin binding domains and the increased expression during conidia germination. Cloning of BC95 coding sequence from the cDNA sample isolated from germinated spores for 4 h in B5 medium with fructose was successful. A His_6-Tag coding sequence was added C-terminally for detection and purification, before the sequence was inserted into the multiple cloning site of different expression vectors.

2.3.4.1. *Schizzosaccharomyces pombe*

The fission yeast *S. pombe* represents a reasonable expression system for BC95 due to the presence of four disulfide bridges in each of the three chitin binding domains. Screening of eight *S. pombe* transformants resulted in no overexpressed bands at 63.1 kDa in silver-stained gels, no detection of an anti-His_6 signal in the western blot and only minor chitinase activity using a dot assay containing glycol chitin (pH 6.9), when cell extracts (data not shown) or the concentrated culture supernatant

were used (Fig. 4C and D). The extracellular protein fractions were then deglycosylated to detect possible covered tags due to protein glycosylation (Fig. 4A and B). CDA activity gel tests on glycol chitin were also carried out at pH 5.0, 7.0 and 9.0. However, no activity was revealed in any of these experiments. All these findings were confirmed in a second experiment.

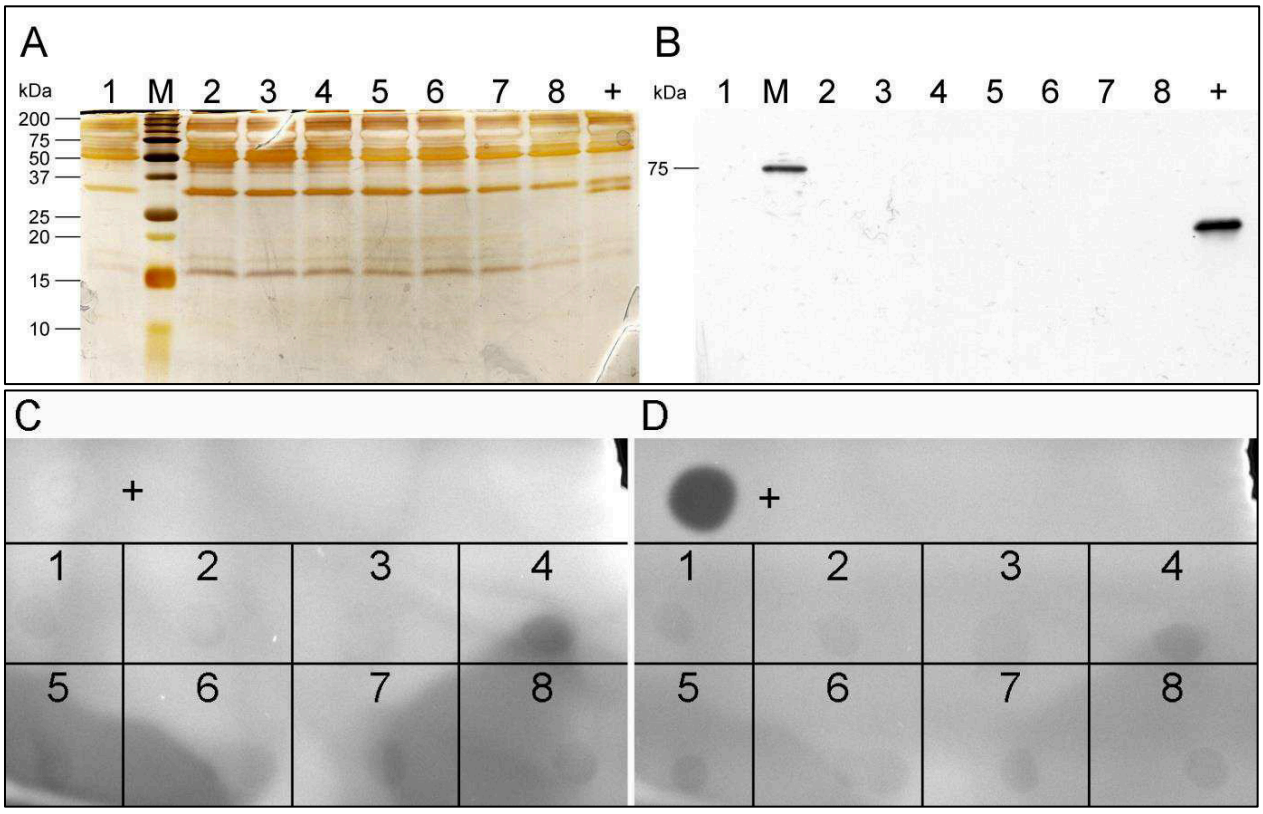

Fig.4: Heterologous production of BC95-His$_6$ in *S. pombe*: analysis of culture supernatants from selected clones 1-8 and positive control (+) **A**: separation of deglycosylated samples by SDS-PAGE (silver stained) and **B**: western blotting (anti-his immunodetection), M: Precision Plus Protein All Blue Standards (Bio-Rad) **C**: CDA activity test using 0.1% glycol chitin gel at pH 6.9 stained with Calcofluor white. Dark spots before depolymerization of chitosan by HNO_2 represent chitinase activity, **D**: spots afterwards are due to CDA activity.

In a second approach, the coding sequence of the His$_6$-Tag was replaced by a StrepII-Tag sequence. *S. pombe* transformants were grown and screened. Five cloneses were chosen for further screenings; however, all five revealed no activity on glycol chitin gels with pH 6.0, 6.9 and 8.0 for all cell fractions tested. Anti-StrepII signals were stronger in deglycosylated than in unmodified samples, but these bands did not correspond with the correct protein size of 63.3 kDa in addition, these signals were also observed in the control (Mock, Fig. 5). Clone 3 showed an additional band of 20 kDa in the culture supernatant (Fig. 5A and B), which could be a tagged degradation product of BC95-StrepII.

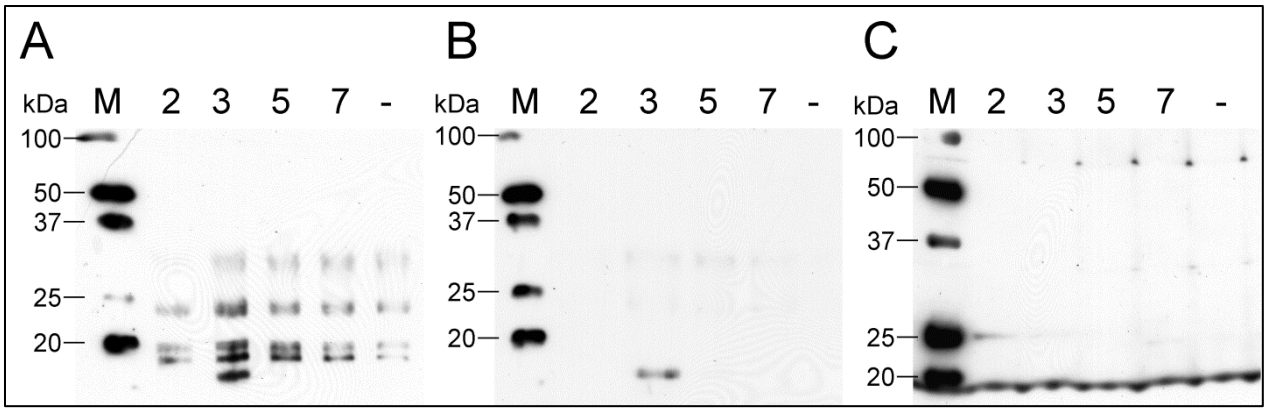

Fig. 5: Heterologous production of BC95-StrepII in *S. pombe* – western blot analysis by Strep-Tactin HRP conjugate, samples of selected clones (2,3,5,7) and negative control strain (-) **A**: deglycosylated culture supernatants, **B**: non-treated culture supernatants and **C**: cell extracts, M: Precision Plus Protein All Blue Standards (Bio-Rad). No signal present for BC95-StrepII (63 kDa).

2.3.4.2. *Escherichia coli*

E. coli was used as expression system, because the production of BC95 was not successful in *S. pombe*. To construct expression plasmids, the coding sequence of BC95-StrepII was cloned into pET22b, once with and once without N-terminal thioredoxin A coding sequence for a more reducing environment. The two constructs were tested simultaneously in *E. coli* Rosetta 2 (DE3) [pLysS-RARE2] expression strain.

Screening of auto-induced expression cultures included cell extracts and pellets, and for some clones cultivation in baffled flasks as well. The comparison of non-induced and induced samples after SDS-PAGE (Fig. 6A) showed no overexpressed bands for BC95 as well as for TrxA-BC95. Figure 6B presents the western blot analysis by StrepII detection with bands at 17 kDa, which is the size of *E. coli* biotin carboxyl carrier protein but lanes with insoluble protein were massively overloaded. Activity gels using the substrate glycol chitin were carried out at 37°C and 20°C (Fig. 6C) and resulted in detection of light chitinase activity for both protein products and all tested clones. A second set of experiments showed the same result that no BC95 enzyme, with and without thioredoxin A fusion, could successfully be purified.

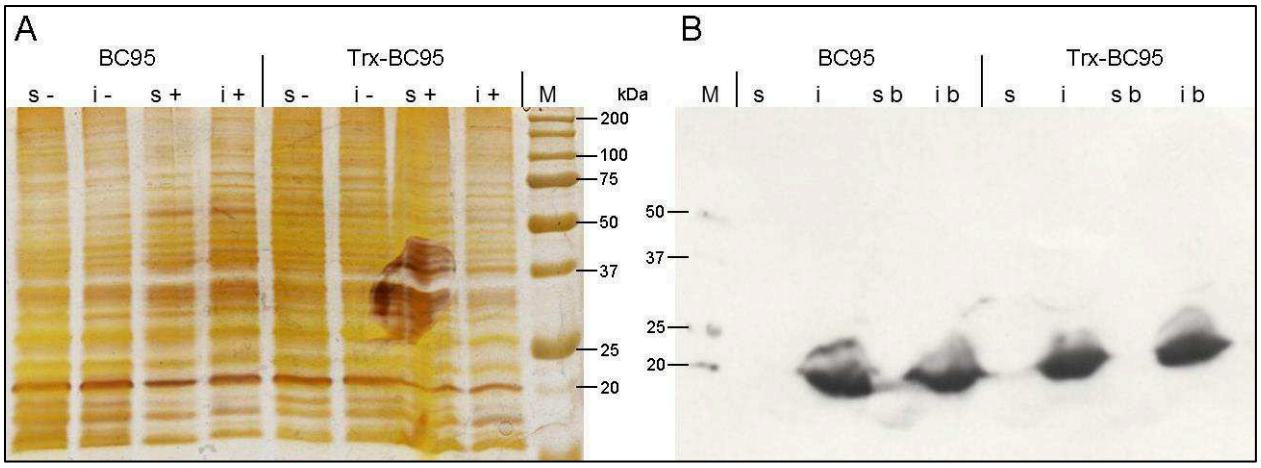

Fig. 6: BC95-StrepII production in *E. coli* Rosetta2 (DE3) [pLys-RARE] with pET-22b::BC95-StrepII (BC95) or pET-22b::TrxA-BC95-StrepII (Trx-BC95), cell extracts analyzed for soluble (s) and insoluble (i) proteins using 12% polyacrylamide gels and Precision Plus Protein All Blue Standards (Bio-Rad) molecular weight marker (M), **A: SDS-PAGE** including auto-induced (+) and non-induced (-) samples, silver-stained, **B: Western blot** with Strep-Tactin HPC conjugate, additional cultivation in baffled flasks (b)

2.3.4.3. *Hansenula polymorpha*

H. polymorpha, belonging to the genus of Pichia (*P. angusta*), is an expression system that combines the advantages of fast growth, high production and the ability of glycosylation. Because previuos trials for successlul protein expression failed, the BC95 gene was codon-optimized and synthesized before multi copy genome integration into *H. polymorpha* was carried out by Artes Biotechnology (Langenfeld, Germany). The company also pre-screened pools of transformants for successful genome integration (Fig. 7A). Western blot analysis revealed transformants producing BC95, but signals of tagged protein were detected in cell extracts only, indicating that secretion was not working properly. Bands at 75 kDa appeared with a higher molecular smear in the same gels. To prove if glycosylation of the proteins caused these results, cell fractions were divided into soluble and insoluble extracts and enzymatically de-N-glycosylated (Fig. 7B). While the two insoluble protein samples appeared to be similar after this procedure, deglycosylation of the soluble cell fraction lead to a shift in the pattern, but not to distinct bands. This result implies that soluble BC95 is N-glycosylated, but may also form aggregates with other proteins. Testing different culture media with varying pH values (5, 6 and 7) and also at lower temperatures for improved protein folding (25°C and 30°C) did not result in better enzyme secretion, except when using the standard medium (YPYNBG) at 30°C (data not shown). Therefore, cultivations for protein production (de-repression) were since carried out at 30°C.

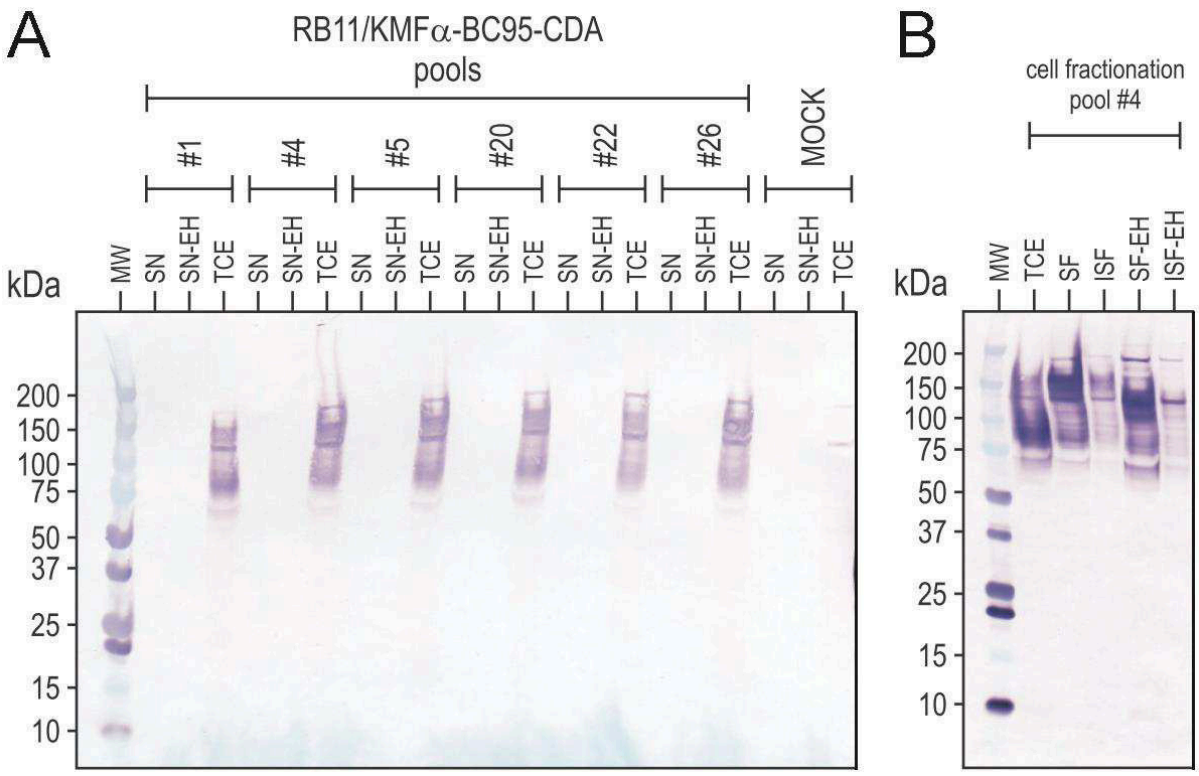

Fig. 7: BC95-StrepII production in *H. polymorpha,* western blot analysis by Artes Biotechnology GmbH (modified), using Strep-Tactin-HRP conjugate (IBA) and chromogenic detection for pools (#) expressing recombinant MFα-BC95. **A**: samples of culture supernatants (SN), de-N-glycosylated supernatants (SN-EH) and total cell extracts (TCE), Mock: strain for negative control, **B**: fractionated samples of pool #4 including soluble (SF) and insoluble fraction (ISF) as well as both fractions treated for deglycosylation (SF-EH and ISF-EH).

After the production of tagged protein was verified by Artes in the soluble cell fraction, I tested cell extracts and supernatants of pools #1, #4 and Mock clone (negative control) for the activity on glycol chitin gels (Fig. 8A and B) and for acetate release using chitosan DA 56%, colloidal chitin and chitin pentamer as substrates. The figure indicates chitinase activity for all samples as well as for the negative control, especially in the culture supernatant, while no CDA activity was observed. For the CDA activity tests, no acetate release could be detected and these results were confirmed by two further experiments.

In a second step, cell fractions were analyzed on SDS-PAGE and western blot (data not shown). In these experiments strep-tagged bands could be identified in the intracellular fractions. However, the Mock strain containing no gene for BC95 produced the same pattern of bands as the pools #1 and #4; hence, these signals were derived from unspecific bindings.

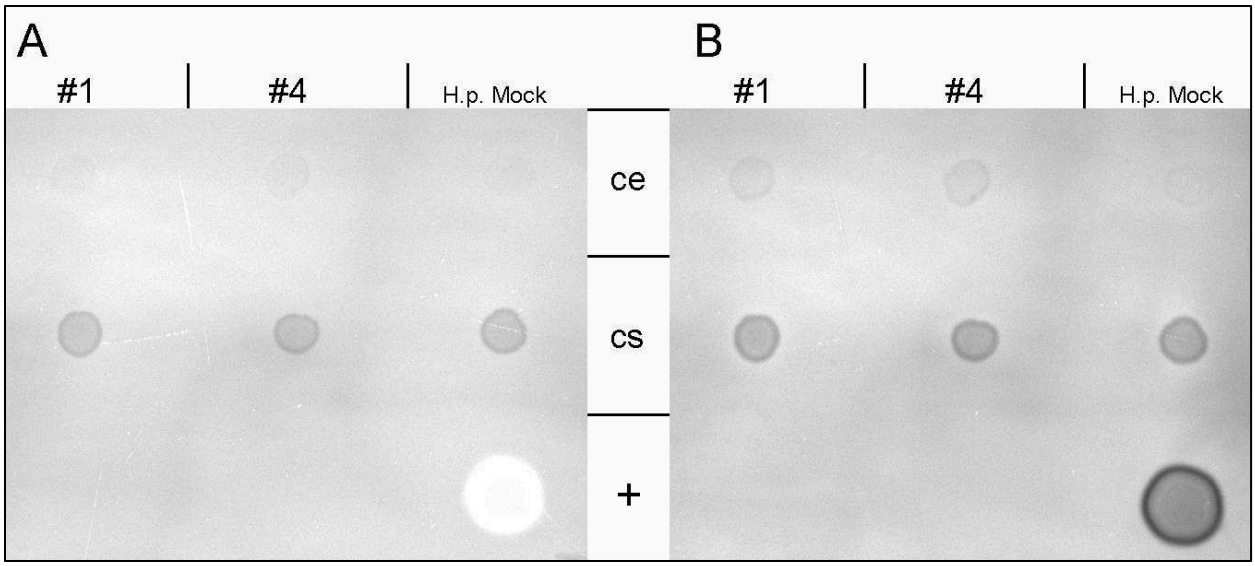

Fig. 8: BC95-StrepII production in *H. polymorpha,* CDA activity assay, analysis of cell extracts (**ce**) and culture supernatant (**cs**) using 0.1% glycol chitin and 25 mM phosphate-borate buffer pH 8.0 for 2 µl samples each and incubation at 37°C, before (**A**) and after depolymerization of chitosan (**B**).

In summary, the heterologous production of the putative *Botrytis cinerea* CDA BC95 in *S. pombe* and *E. coli* was not successful, but could be achieved by using the *H. polymorpha* expression system. Despite using the secretion signal MF-α1, the protein remained mostly intracellular, but soluble and probably glycosylated. Chitin deacetylase activity could not be confirmed on different polymeric and oligomeric, chitinous substrates.

2.4. Discussion

The existence of chitin deacetylases in combination with chitin binding domains is a hint towards enzymes that are capable of deacetylating crystalline chitin. So far, chitosans are commercially produced by chemical deacetylation of chitin only, because known CDAs act on the surface of crystalline chitin only but fail to unwind the crystal for further or even complete deacetylation [Aye et al., 2006]. Although some insect sequences have been discussed to possess these domains and abilities [Dixit et al., 2008], enzymatic activity of such CDAs, especially towards crystalline substrates, have never been addressed before. It has been demonstrated that fungal CDAs differ in their overall properties including the modes of action [Tsigos et al., 2000]; hence, CDAs including CBDs could vary from enzymes just solely possessing the catalytic domain in properties and substrate preferences as well. Novel enzymes acting on crystalline chitin might give the opportunity to create new chitosans

offering potentially non-random patterns of acetylation, which cannot be archived by chemical methods [Tsigos et al., 2000]. Fungal chitosans occurring in nature are usually generated by CDAs [Kaur and Dhillon, 2014] and are biologically active, e.g. in plant systems [Hadwiger, 2013], where they amongst other functions play a role in plant-pathogen interactions [El Gueddari et al., 2002]. Thus, for plants secreting sequence specific chitinases and sensing chitin, the PA is more probably than not important. In this study I identified several putative CDAs containing one or more CBDs within the same coding sequence belonging to *Botrytis cinerea*, a model plant pathogen. Their expression during conidia germination and the production trials of recombinant BC95 in three different systems are shown here.

2.4.1. *Botrytis cinerea* putative chitin deacetylases

The bioinformatic analysis of the six putative CDA genes of *B. cinerea* revealed that all sequences differ in length and domain architecture (Fig. 1). A comparison of these sequences showed a high similarity in peptide sequence within the domains. Genomes of other fungal species also contain CDA genes including CBDs that are similar to the *B. cinerea* genes in sequence as well as in domain structures, for example the closely related *Sclerotinia sclerotiorum* [Amselem et al., 2011]. It was shown that filamentous fungi possess more chitinase genes than yeasts, because this lifestyle requires more isoenzymes due to the complex morphology [Karlsson and Stenlid, 2008]. This might also be the case for CDAs, especially those expressed by plant pathogens since an important role of pathogenic CDAs in plant-pathogen-interaction has been reported for several species [El Gueddari et al., 2002]. The combination of polysaccharide deacetylase with chitin binding domains of different types seem to be evolutionally invented early and multiple times, because it can be found in many species, even in bacteria [Kadokura et al., 2007]. However, the occurrence of CDAs with CBM18 is limited to ascomycetes as revealed by my database research.

Here I present that that all six of the CDA genes identified in *B. cinerea* are expressed at 4 h of conidia germination (Fig. 2). The germination in medium containing fructose as C-source on a glass surface, which is imitating the hydrophobicity on a plant leaf, was consistent to reported experiments by Doehlemann, et al. [2006]. Spores incubated in bi-distilled water did germinate slowly but mycelium did not grow under these conditions. These samples might represent an earlier time point in development or starving conditions due to the lack of nutrients. Consistent with our result, it is reported in a recent paper that BC95 (referred to as *cda1*) is strongly upregulated in early time points (1 and

4 h) of germination; therefore it is proposed to play a key role in germination before germ tubes emerge [Leroch et al., 2013]. In my experiments also the five remaining putative CDA genes were expressed in fructose medium as well as in bi-distilled water, except for BC1G_09146 that was absent in the fructose medium. As many changes in cell wall composition happen during development [Cantu et al., 2009], each of these enzymes may have its own task.

To investigate if any CDA activity is detectable during conidia germination, I used a dot gel assay with glycol chitin as substrate and a pH range from 6 to 8. Ascomycetes are known to mainly secret their CDAs into their growth media [Zhao et al., 2010], but active enzyme could also be present intracellular or in the cell wall. So the concentrated culture supernatants as well as fractions of cell extracts and cell walls have been tested, but CDA activity tests failed to present active enzyme. Although the activity gels are usually a very reliable, highly sensitive and easy method for CDA activity screening, it cannot be excluded that these enzymes are not acting on glycol chitin, because it is not a natural substrate. Furthermore the quantities of CDA proteins might have been too little compared to the total protein amount in the tested samples. Assuming that these CDAs are specialized on fungal cell walls [Zhao et al., 2010], they might strongly be bound to the chitin by the CBDs, since macroscopically irreversible binding has been reported for a cellulose binding domain [McLean et al., 2002]. Possible CDA activity in the pelleted mycelium (Fig. 3) might then be masked by the chitinase activity also present in these samples and causing dark spots as well on the activity gels [Trudel and Asselin, 1989].
Shortly after our findings the early secretome of *Botrytis cinerea* was investigated, but none of the putative CDAs was found after 16 h inoculation of conidia in different media shaking cultures [Espino et al., 2010]. This result leads to the assumption that direct isolation of the enzyme from the fungus might not be easy. To investigate if the putative proteins are real CDAs, the strategy of heterologous production of one of them was followed, like it was done before for a CDA from *Flammulina velutipes* [Yamada et al., 2008].

2.4.2. Heterologous production of BC95

BC95 was chosen for heterologous production because it shows a unique domain architecture containing three CBDs and an increased expression during spore germination. The production host *S. pombe* (fission yeast) offers the possibility to get properly folded, secreted glyco-protein, especially of

proteins rich in disulfide bridges, as typical for CBDs. In the present study no recombinant protein was detected in any of the experiments (Fig. 4). CDA pH optima have been reported to range from 4.5 to 12 [Zhao et al., 2010]. However, no activity on glycol chitin was found under several pH conditions tested. In contrast to other fungi, *S. pombe* cell walls are considered to contain no chitin and only small amounts of N-acetylglucosamine in their polysaccharides [Horisberger et al., 1978]. Nevertheless, heterologous expression of a chitinase and a chitosanase, respectively, changed the cell morphology [Shimono et al., 2002]. This is not expected for CDAs, though the fission yeast seems to secret an own chitinase into the culture supernatant, as light activity was detected.

The genome of *S. pombe* contains only one chitinase gene, which is to my best knowledge not further characterized. This is a clear disadvantage fof using this host for the production of chitin and chitosan modifying enzymes, as the presence of organism own substrate degrading enzymes has to be excluded. Because the purification of heterologously expressed and also tagged protein via affinity chromatography is usually carried out directly after production and thus, the organisms own chitin modifying enzymes are removed, the presence of *S. pombe* chitinase was regarded to be not problematic for the enzyme characterization that followed afterwards.

In further experiments the C-terminal His_6-tag coding sequence of BC95 was exchanged to a StrepII-tag coding sequence [Schmidt and Skerra, 2007]; taking into account that the bivalent cation affinity may have caused problems for enzyme activity. By solving the crystal structure of a *C. lindemuthianum* CDA, which was heterologously produced in *P. pastoris* including a His_6-tag, Blair and colleagues [2006] found an intermolecular interaction between the His_6-tag and the active center containing the Zn^{2+} cofactor. By using the StrepII-tag for detection and purification, this kind of interaction is excluded. The new *S. pombe* transformants were analyzed by western blots (Fig. 5), where multiple bands could be found, especially with the deglycosylated culture supernatants. However, no signal corresponded to the correct molecular weight of 63 kDa and again no CDA activity at different pH values was detected. A band with the size of about 20 kDa is only present in one clone, which might be caused by degradation of the full BC95, as the StrepII-tag is located C-terminally, or by unspecific interactions. These results brought us to the conclusion that the successful production in *S. pombe* is highly questionable, so the use of a more straightforward expression system was considered.

Escherichia coli is one of the major used prokaryotic expression system. Many eukaryotic proteins have successfully been expressed by using *E. coli* expression systems, and today, many specialized tools for several challenges during expression of difficult proteins are available. I used the Rosetta 2 (DE3) strain carrying the pLysS-RARE2 plasmid for avoiding any matters with codon usage [Novy et al., 2001] as well as a variant with *E. coli* innate thioredoxin A as N-terminal fusion with BC95. Cytoplasmic co-expression of thioredoxin A or fusion to the target protein can create a more reducing environment for the linking of the disulfide bridges; hence, increase the yield of correctly folded recombinant protein [Collet et al., 2005; Lavallie et al., 1993]. Checking the constructs with and without thioredoxin fusion, auto-induced cultures were analyzed and non-induced cultures were used as expression controls in the first experiment. No overexpressed protein could be identified by SDS-PAGE (Fig. 6A) neither in soluble nor in insoluble intracellular fractions. In the second experiment baffled flasks were used to increase oxygen level during cultivation. In the western blot analysis (Fig. 6B) signals of the intracellular *E. coli* biotin carboxyl carrier protein could be detected, but no BC95 or TrxA-BC95 with the size of 64 kDa and 76 kDa, respectively. The absence of CDA activity tested on glycol-chitin was clear, because no chitinase activity disturbing the assay was present in the *E. coli* samples in contrast to the yeast earlier. A CDA from *Aspergillus nidulans* was successfully produced with the *E. coli* pET-vector system, but the enzyme had to be renatured from inclusion bodies after induction by IPTG and is a small protein without CBDs [Wang et al., 2009]. In the present study, BC95 was not produced at all and may not only need assistance for correct folding, but also glycosylation for solubility and function like the majority of fungal CDAs [Zhao et al., 2010].

By going back to a yeast system, two major improvements were implemented: The BC95 coding sequence was optimized for heterologous expression in the new host and synthesized including the StrepII-tag, and generation of stable transformants of an *H. polymorpha* industrial strain was executed by Artes Biotechnology GmbH. Similar to the fission yeast, *H. polymorpha* is engineered for correct folding, high production and enhanced secretion of recombinant proteins, but in contrast to *P. pastoris* the production cultures are methanol-free [Gellissen, 2000]. The presence of recombinant protein could be shown by Artes' first screening with bands on western blot at and above 75 kDa, which were missing in the corresponding controls (Fig. 7A). The reason why the protein stays intracellular is probably up to a lacking or incomplete processing of the pre-pro-peptide, which is in accordance with the size of BC95 including the pre-pro-sequence of 72 kDa. However, the production of soluble BC95

turned out to be successful. CDA activity was tested with 4 different chitinous substrates, but could not be detected in any case, while replication of Artes' results was also not achieved.

Yeast expression systems have the advantage of glycosylation ability, but are also known to over-glycosylate [Mattanovich et al., 2014]; hence the glycosylation of BC95 as seen by western blot analysis may be too much or not appropriate for enzyme function (Fig. 7). Pre-pro-BC95 possesses three N-glycosylation sites in the MF-α1 sequence, additionally to the six sites of BC95, which together account for the lesser protein mobility during SDS-PAGE. Unfortunately the de-N-glycosylation is only accomplishable under denaturating conditions, so deglycosylated samples cannot be tested anymore for their activity. As stated before, a broad range of pH optima have been reported for CDAs and although one could expect to find a little activity at medium pH values, this has not been the case. In spite of the combination of a PDD with CBDs, it might thus be possible that BC95 is no CDA at all. Enzymes containing PDDs of the carbohydrate esterase family 4 (CE4, CAZY database) include chitooligosaccharide deacetylases, xylanases and acetylxylan esterases, some with overlapping substrate specificities [Caufrier et al., 2003]. Therefore, other substrates like xylan should be tested in further experiments. For this purpose, the production of BC95 has to be optimized either by cultivation and purification from cell extract or ideally by improvement of the secretion.

The genome of *B. cinerea* contains an interesting set of six putative CDA genes, which are all expressed during conidia germination. To analyze enzymatic function the gene of BC95 with three N-terminal CBDs was cloned for heterologous production in the three different expression hosts *S. pombe, E. coli* and *H. polymorpha.* Production of recombinant protein was only achieved with *H. polymorpha,* but CDA activity was not detected. Whether this is due to incorrect processing and lacking secretion or a different enzymatic function has to be addressed in further studies.

Chapter 3

A chitin deacetylase from *Podospora anserina* with two carbohydrate-binding domains produces chitosans with non-random patterns of acetylation

This chapter will be submitted after slight modifications as a manuscript for publication with the following co-authors: Andreas Kranz and Bruno M. Moerschbacher. Dr. Andreas Kranz from Artes Biotechnology GmbH was responsible for the generation of *Hansenula polymorpha* production strains (sections 3.2.2 and 3.3.2) and Prof. Dr. Bruno M. Moerschbacher is the supervisor of this doctoral thesis.

Abstract

Chitosan is a structurally-diverse biopolymer that is commercially derived from chitin by chemical processing, but chitin deacetylases (CDAs) offer a sustainable and potentially more controllable approach allowing the production of chitosans with tailored biological activities. We investigated the chitin deacetylase from *Podospora anserina* (PaCDA) and found that it was closely related to *Colletotrichum lindemuthianum* CDA in the catalytic domain, but was unique due to the presence of two chitin-binding domains. We produced recombinant PaCDA in *Hansenula polymorpha* and found that the purified 35.5-kDa protein was active against glycol chitin, chitin oligomers with a degree of polymerization ≥ 2, chitosans and colloidal chitin. The pH optimum against this latter substrate was 10.5, and the enzyme remained stable within the pH range 5–8.5. The optimal temperature was 55°C and the half-life at 80°C was 11.8 ± 0.6 min. PaCDA was not inhibited by EDTA or acetate and the cofactor was Zn^{2+}. These data show that the catalytic domain of PaCDA is both structurally and functionally similar to *C. lindemuthianum* CDA. We also studied the mode of action on chitosans by enzymatic/MALDI-TOF MS fingerprinting and NMR using chitosans with the same degrees of polymerization and acetylation. Chemical processing and enzymatic deacetylation with PaCDA generated distinct patterns of acetylation (PAs). These chitosans were also compared in terms of their biological activity towards *Oryza sativa* cell cultures. There were no differences found in this case of oxidative burst assay, but we have achieved the enzymatic production of chitosans with non-random PAs.

3.1. Introduction

Chitin is a linear polysaccharide of β-1,4-linked N-acetylglucosamine (GlcNAc) units which was first isolated from fungi by Henri Braconnot more than 200 years ago [Muzzarelli et al., 2012]. Later, it was recognized as the major component of arthropod exoskeletons and now, shrimp shell waste is the main source of this compound [Vázquez et al., 2013]. The presence of many intermolecular hydrogen bonds gives rise to an insoluble fiber that may exist either in the α- or β-form, depending on the source and orientation of the chains [Pillai et al., 2009].

Chitosan is a structurally-diverse family of derivatives of chitin that can be produced by the release of acetate, resulting in polymers containing glucosamine (GlcN) and GlcNAc units. The amino groups can be protonated under slightly acidic conditions, which makes chitosan more water-soluble than chitin. The solubility and structural diversity of chitosans render them more useful for downstream applications [Ravi Kumar, 2000; Shukla et al., 2013] including water treatment [Bhatnagar and Sillanpää, 2009], medical applications like wound dressing [Jayakumar et al., 2011] or drug delivery [Hu et al., 2013], and plant protection [El Hadrami et al., 2010].

The properties of chitosans can be described by three parameters: the degree of polymerization (DP), the degree of acetylation (DA) and the pattern of acetylation (PA), the latter referring to the sequence of GlcN and GlcNAc units. While we have a basic understanding of structure-function relationships among chitosan molecules concerning DP and DA, little is known on the influence of PA, which based on theoretical considerations, we have postulated to also influence biological activity, especially if the chitosan is cleaved *in situ* by a sequence-specific chitosan hydrolase [El Gueddari et al., 2007]. The role of PA has been difficult to address due to the absence of chitosans with non-random PAs and the lack of suitable analytical methods. Chemically-produced chitosans appear to always display random PAs [Weinhold et al., 2009b] whereas enzymes known as chitin deacetylases (CDAs, EC 3.5.1.41) may have the potential to generate non-random PAs [Tsigos et al., 2000].

CDAs have been identified in bacteria [Kadokura et al., 2007; Li et al., 2007], insects [Dixit et al., 2008] and fungi [reviewed in Zhao et al., 2010] including *Mucor rouxi* [Kafetzopoulos et al., 1993]*, Aspergillus nidulans* [Alfonso et al., 1995], *Saccharomyces cerevisiae* [Martinou et al., 2002], *Rhizopus circinans* [Gauthier et al., 2008] and *Penicillium oxalium* [Pareek et al., 2012]. The CDA from *Colletotrichum*

lindemuthianum (ClCDA) is the most widely studied [Tokuyasu et al., 1999; Tsigos and Bouriotis, 1995]. CDAs from different fungi modify their substrates in different ways depending on their role in the fungus, acting processively or in a non-processive mode of action [Tsigos et al., 2000]. The zygomycete *M. rouxii* has an intercellular CDA required for cell wall formation that is believed to work processively by a multiple attack mechanism [Davis and Bartnicki-Garcia, 1984; Tsigos et al., 1999]. In contrast, the plant pathogen *C. lindemuthianum* produces an extracellular non-processive CDA [Tokuyasu et al., 2000a]. *Puccinia graminis* f. sp. *tritici* converts its cell wall chitin into chitosan while penetrating the host in order to escape plant defense responses [El Gueddari et al., 2002]. Despite their name, all CDAs analysed so far show only minimal activity against polymeric chitin in vitro unless the crystallinity is broken by pretreatments generating amorphous/colloidal or superfine chitin [Win and Stevens, 2001]. This may indicate that in vitro, enzymatic deacetylation is restricted to the surface of insoluble chitins [Aye et al., 2006]. The lack of enzymatic activity of fungal CDAs towards polymeric chitin may reflect their physiological role when acting on the nascent chitin chain emerging from the chitin synthase before the formation of chitin crystals. However, it may also simply be owed to the low number of fungal CDAs investigated so far which by no means reflects the broad diversity of known genes putatively coding for CDAs.

Many chitinases can degrade chitin crystals completely. Like other carbohydrate-active enzymes they often feature a modular structure featuring catalytic domains and carbohydrate-binding modules (CBMs) [Boraston et al., 2004]. These non-catalytic modules are characterized by their unique fold and carbohydrate-binding activity. CBM family 18 (CAZY database) also known as the type 1 chitin-binding domain (CBD, PFAM 00187) is characterized by eight conserved cysteine residues that form four disulfide bonds [Beintema, 1994]. This domain also occurs without an accompanying catalytic domain in chitin-binding proteins and lectins such as wheat germ agglutinin [Wright et al., 1991]. CBDs help chitinases to act on insoluble chitin polymers [Blaak and Schrempf, 1995; Iseli et al., 1993], and we therefore assumed that they may also do likewise for CDAs, although active CDAs with CBDs have not been described thus far.

Here we describe the discovery, heterologous expression and analysis of a novel CDA containing two chitin binding domains in the genome of *Podospora anserina,* a filamentous ascomycete living as a saprophyte on herbivore dung which is considered a model organism for the genetic and molecular

analysis of senescence/cell aging [Espagne et al., 2008]. We used this enzyme to produce chitosans with non-random PAs and to explore their biological activities, and found that the enzyme was more active against chitosans than crystalline chitin but was able to generate non-random PAs.

3.2. Materials and methods

3.2.1. Bioinformatics

The Pfam database (http://pfam.sanger.ac.uk) was accessed in May 2010 and scanned for protein architectures containing a polysaccharide deacetylase domain (PDD, PF01522) and the "Chitin_bind_1" CBD (PF00187) [Finn et al., 2014]. The resulting list was supplemented with fungal sequences from *C. lindemuthianum, M. rouxii, R. circinans, A. nidulans, P. graminis* and *S. cerevisiae* (CDA1 and CDA2), whose CDA activity was reported in literature [Alfonso et al., 1995; Gauthier et al., 2008; Kafetzopoulos et al., 1993; Mishra et al., 1997; Tsigos and Bouriotis, 1995]. We used ClustalW2 to carry out several multiple sequence alignments (http://www.ebi.ac.uk/Tools/clustalw2) initially using the parts annotated as PDD to identify putative CDAs, but later also with full-length sequences [Larkin et al., 2007]. SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) was used to identify signal peptides [Bendtsen et al., 2004], and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) to predict potential N-glycosylation sites [Blom et al., 2004].

3.2.2. Production and purification of PaCDA

The *Podospora anserina* protein sequence (PaCDA, GeneBank: CAP60162) was sourced from the NCBI database (http://www.ncbi.nlm.nih.gov) and provided to GeneArt® (Thermo Scientific, Germany) for gene optimization and synthesis. We fused the PaCDA gene C-terminally to a StrepII-Tag coding sequence [Schmidt and Skerra, 2007] using two phosphorylated primers coding for one half of the tag each (5'-pho-CAATTTGAAAAGTAGTAAGGATCCGGTACCTGGAGC-3' and 5'-pho-AGGATGAGAC CAGTTACAGTTGCCGAAGGCTCTC-3', StrepII sequence underlined) and amplifying the whole plasmid. The PCR template pMA-T::PaCDA was then digested using restriction enzyme *Dpn*I followed by gel-extraction of pMA-T::PaCDA-StrepII and recirculation of the plasmid. The uracil-auxotrophic (ura3) *H. polymorpha* host strain RB11 [Zurek et al., 1996] was chosen for high-level production of PaCDA. To construct expression plasmids, the optimized sequence of PaCDA was provided to Artes

Biotechnology GmbH (Langenfeld, Germany) where it was upstream extended with the pre-propeptide MF α1 coding sequence of *S. cerevisiae* [Kurjan and Herskowitz, 1982] in order to achieve secretion, and the construct was cloned into pFPMT121 [Degelmann et al., 2002]. This plasmid contains the *FMD* (formate dehydrogenase) promoter and the *MOX* (methanol oxidase) terminator as transcriptional control elements for inserted genes. Preparation of competent cells and transformation were carried out as described before [Klabunde et al., 2007]. To promote mitotically stable integration of multiple expression plasmid copies into the *H. polymorpha* genome, uracil-prototrophic transformants were subjected to repeated cycles of growth under selective and nonselective conditions (passaging and stabilization). Stabilized transformants were cultivated in glycerol-containing medium to induce expression (YPYNBG, 1% yeast extract, 2% peptone, 0.14% yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulphate, 2% glycerol, and 0.1 M potassium phosphate buffer pH 6.0) for 70 h at 30°C.

We initially screened pools of *H. polymorpha* clones for amounts of secreted product present in cell-free culture supernatants, estimated by Western dot blot using Strep-Tactin-HRP conjugate and chromogenic detection. Then we identified individual clones and screened for amounts of active enzyme on CDA activity gels containing glycol chitin (prepared as previously described by Trudel and Asselin, 1989). The samples of culture supernatant were applied as drops of 1 µl each onto the gels (dot gels). These were prepared with 12% polyacrylamide, BisTris buffer pH 7.0 and 0.1% (w/v) glycol chitin, incubated overnight at 37°C, followed by staining with Calcofluor White (Fluorescent Brightener) and chemical depolymerization of deacetylated chitin using HNO_2 as described by Trudel and Asselin (1990). Afterwards, the transformants showing high productivity were selected for subsequent experiments.

For enzyme production on larger scale 2-l flasks with 200 ml YPYNBG were inoculated with 4 ml of pre-culture and incubated for 70 h at 30°C and 180 rpm. The cell-free culture supernatant was concentrated and rebuffered to 20 mM triethanolamine (TEA) pH 8.0 and 400 mM NaCl by ultrafiltration through 10-kDa molecular weight cut-off filters in a 400-ml stirred device. The protein was purified by StrepTactin affinity chromatography on a Qiagen Strep-Tactin Superflow 1 ml column in an ÄKTAprime plus FPLC system (GE) according to the manufacturers' protocols. Purified PaCDA was stored at -20°C in 50 mM ammonium carbonate buffer pH 8.5 and 50% glycerol.

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) was carried out as described [Laemmli, 1970], using gels containing 12% (w/v) polyacrylamide and Coomassie Brilliant Blue

staining. The semi-native PAGE was carried out like the SDS-PAGE, but without boiling and addition of β-mercaptoethanol to the sample, at 4°C and followed by washing steps for renaturation in BisTris buffer pH 7.0 with Triton X-100 and a glycol chitin overlay gel as previously described [Trudel and Asselin, 1990], then CDA activity was detected as described above.

3.2.3. Substrates

Chitosan DA 0% (DP≈1540), α- and β-chitin were kindly donated by Gillet-Matani Chitosan (France/ India). We prepared reacetylated chitosans as previously described by Vachoud et al., (1997), using molar ratios of acetic anhydride in acetic acid/1,2-propanediol/water solution. Colloidal chitin was produced from β-chitin by slightly modifying the protocol described by Hsu and Lockwood (1975). Chitin oligomers were purchased from Seikagaku Corporation (Japan) and Megazyme (Ireland).

Deacetylation of chitosans with different DA values ranging from 9% to 60% and DP values ranging from about 600 to 1500 was tested by adjusting the substrate concentrations to 0.05 g/l acetate groups. The 330-µl samples were incubated with 0.075 µM PaCDA at 37°C for 120 h in 50 mM TEA buffer pH 8.5 and 50 mM NaCl. The CDA activity was determined by measuring acetate release [Martinou et al., 1995] using an acetic acid assay kit according to the manufacturer's recommendations (R-Biopharm AG, Darmstadt, Germany) but downscaled for 96-well mircotiter plates. Samples were measured in triplicates and acetate concentrations were calculated against a set of standards. Chitin oligomers and monomer (N-acetylglucosamine) from DP 1 to 6 (0.1 g/l) were incubated with 0.12 µM PaCDA at 37°C for 120 h in 23 mM TEA buffer pH 8.5. In addition to the acetate assay, samples were also analyzed by mass spectrometry (MS, see section below). Activity of PaCDA on colloidal, α- and β-chitin was tested using 1 g/l substrate and 0.246 µM enzyme in 400 µl of 49 mM TEA buffer pH 8.5 with 49 mM NaCl and incubating at 37°C and 180 rpm for 120 h. After centrifugation at 15,700 g and 4°C for 10 min, the products in the supernatant were analyzed as described above.

3.2.4. Characterization of PaCDA

Colloidal chitin (0.1 g/l) was used as substrate for 0.123 µM PaCDA in 400-µl sample volumes shaken in an Eppendorf thermomixer at 750 rpm. The reaction was stopped by removing the insoluble substrate by centrifugation (15,700 g, 4°C, 10 min) before the CDA activity was determined by measuring acetate release from the supernatant [Martinou et al., 1995]. The impact of pH values 4–12

was tested by incubating samples at 37°C for 17 h in 25 mM Teorell-Stenhagen-buffers (citrate, phosphate and borate). The stability within this range was tested by pre-incubating PaCDA at 37°C for 24 h in 50 µl 40 mM Teorell-Stenhagen-buffers, followed by the addition of the substrate in 37.5 mM TEA buffer pH 8.5 and 37.5 mM NaCl and incubating at 37°C for 17 h.

The impact of temperatures over the range 4–90°C was tested by incubating samples for 1 h in 50 mM TEA buffer pH 8.5 with 50 mM NaCl. The temperature stability was determined by incubating 5-µl aliquots of enzyme solution (1 g/l) in a thermocycler at 80°C for up to 2 h, and then processing the samples as discussed above but incubating at 37°C for 17 h. The half-life was calculated by plotting time against activity relative to untreated enzyme using SigmaPlot (Systat Software Inc.) and calculating the fit to the exponential decay (triple, 7 parameter) curve.

The influence of bivalent cations (1 and 10 mM, as chloride salts, listed in Table 1), EDTA (1 and 10 mM) and β-mercaptoethanol (0.5% and 5%) was tested by pre-incubation with the enzyme at room temperature or at 95°C for 5 min in 39.5 mM TEA buffer pH 8.5 with 39.5 mM NaCl, followed by substrate addition and incubation as described above.

Total reflection X-ray fluorescence analysis (TXRF) was carried out on a S2-PICOFOX instrument (Bruker AXS, Berlin, Germany) with an air-cooled molybdenum anode for X-ray generation as previously described [Telgmann et al., 2011]. For the determination of the bivalent cations iron, cobalt, nickel, copper and zinc as possible co-factors, aliquots of the purified PaCDA samples were mixed with the same volume of the 10 mg/L gallium standard solution, placed on quartz glass disks sample carriers and evaporated to dryness. The analysis was carried out by signal integration over 200 seconds with excitation settings at 50 kV and 750 mA, followed by quantification using the Bruker Spectra software (version 6.1.5.0) based on the known concentration of the internal gallium standard.

Acetate inhibition was tested by incubating 0.25 g/l chitin pentamer with 0.615 µM PaCDA and 100 mM ammonium acetate at 37°C for 21.5 h in 50 mM ammonium carbonate buffer pH 8.5. Buffer was removed by three drying and resolving cycles in a centrifugal concentrator at 60°C before MS analysis (see below).

3.2.5. Mode of action

To analyze the mode of action on polymers, 500 mg reacetylated chitosan DA 60% (as described above) was used as substrate for 300 µg PaCDA and incubated at 37°C and 120 rpm for 3 h in 1-l flask with 500 ml of 25 mM TEA buffer pH 8.0. We monitored the resulting DA by analyzing samples

for acetate release (as described above) to stop the reaction at around DA 30% by heating at 90°C for 10 min. During the process of deacetylation the chitosan was partly precipitating and at the end, 25% ammonia solution was added for full precipitation at pH 9-10. The precipitate was washed twice in alkaline, then twice again in neutral distilled water to remove enzyme and buffer, and freeze-dried in the last step. The final DA of PaCDA-treated chitosan was determined by ^{1}H-NMR (see section below).

The pattern of acetylation was analyzed by enzymatic/mass spectrometric fingerprinting, carried out using a chitinolytic enzyme from *Alternaria alternata* known as chitinosanase [Beneteau et al., in preparation]. PaCDA-treated and chemically reacetylated chitosan DA 30% (10 µg) was hydrolyzed by 10 ng enzyme in 20-µl samples at 37°C in 80 mM ammonium acetate buffer pH 4.2 in an over-night reaction. Hydrolysates were directly scanned for resulting chitosan oligomers using mass spectrometry.

3.2.6. Mass spectrometry (MS)

Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) analysis was carried out using 355 nm SmartBeam^{TM} NdYAG-laser and Bruker Daltonics Autoflex Speed (Bremen, Germany). Samples were prepared by mixing 0.7 µl of enzyme products (≈0.1 g/l) with 0.7 µl of 2,5-dihydroxybenzoic acid (DHB) matrix solution (10 g/l in a 1:1 acetonitrile:H_2O mixture) on target and blow-dried. For MS^2 experiments 3-aminoquinoline (3-AMQ) was used in optimal conditions described by Rohmer et al., [2010], because this matrix substance also acts as derivatizing agent tagging the reducing end and thereby enables oligosaccharide sequencing after post-source decay (PSD) fragmentation. Measurements were carried out by the MS service of the Institute of Organic Chemistry at WWU Münster. The spectra were evaluated using the latest available version of Mmass [Strohalm et al., 2010]; after base line correction, the monoisotopic peaks of chitooligosaccharide sodium ions were annotated according to their composition of GlcNAc (A) and GlcN (D).

3.2.7. Nuclear magnetic resonance spectroscopy (NMR)

DAs of chitosans were determined by dissolving ≈2 mg each in about 500 µl of D_2O with 2 µl of DCl, followed by two freeze-drying and resolving cycles to remove H_2O, and recording ^{1}H-NMR spectra at 400 MHz (Bruker Avance II 400, Bruker Corporation, USA) as described previously by Hirai et al.

[1991] and Vårum et al. [1991] and validated by Lavertu et al. [2003]. Measurements were carried out by the NMR service of the Institute of Organic Chemistry at WWU Münster and spectra were evaluated using MestReNova Software v. 9.0 (Mestrelab Research S.L, Spain).

For determination of the PA by ^{13}C-NMR diad analysis [Varum et al., 1990] I dissolved and depolymerized chitosans as described by Kumirska et al. [2009] and Weinhold et al. [2009], using ≈100 mg of each sample in 1.4 ml. Spectra were recorded at 600 MHz and 70°C (Agilent DD2 600, Agilent Technologies, USA) and evaluated with MestReNova Software v. 9.0 (Mestrelab Research S.L, Spain) analogous to the literature specified above for spectrums with a good signal to noise ratio. A manual phase correction on PH0 and PH1 and an exponential apodization of the spectra in the limits 0.3 - 0.85 Hz or Gaussian apodization until 7 GB (Hz) was carried out before baseline correction using the Bernstein polynomial fitting with a polynomial order up to 4. For integration of the diad frequencies of the C5 carbon atom, the MestReNova function for Lorentzian Gaussian deconvolution was applied by setting a fit region between 74.5 and 76 ppm using width constraint 0.1 - 100 Hz and a maximum number of 100 fine iterations was fixed. The P_A value was calculated according to equation 1 with the different fractions (F_{DD}, F_{AA} and $F_{AD}^* = F_{AD} + F_{DA}$) representing the relative intensities of the four possible sequences in percent [Kumirska et al., 2009; Weinhold et al., 2009b].

Eq.1

$$P_A = \frac{F_{AD}*}{(2 \times F_{AA}) + F_{AD}*} + \frac{F_{AD}*}{(2 \times F_{DD}) + F_{AD}*}$$

3.2.8. Biological activities

Oxidative burst assay with suspension cultured *Oryza sativa* cells was carried out by slightly modifying the protocol described by Melcher and Moerschbacher [in preparation]. *O. sativa* cells were obtained and cultivated as described [Ortmann et al., 2006] and used for the assay 3-4 days after re-inoculation. Preparation of 96-well plates was carried out by weighing 1.2 g rice cells in 30 g assay medium, pipetting 50 µl cell suspension per well while shaking gently and adding 150 µl assay medium for an optimal cell concentration of 0.02 g/l. The cells were pre-treated with L012 luminol derivative (Wako Chemicals, Neuss, Germany) by adding 20 µl of a 0.1 g/l L012 solution in 50 mM potassium phosphate buffer pH 7.9, before they were incubated at 26°C and 120 rpm for 4 h. Prior to

eliciting, 15 µl horseradish peroxidase type II (1 g/l, purchased from Sigma-Aldrich, Hamburg, Germany) was added as a catalyst to each well to enhance chemiluminescent reaction. To test possible elicitor substances, 30 µl samples of 1 g/l chitosan solutions each were added to the cells by a multichannel pipette before recording the emitted light for 1 s every 90 s (40 data points) in a microplate luminometer (Luminoskan Ascent, Thermo Scientific, Waltham).

3.3. Results

3.3.1. Bioinformatics

We searched for putative CDAs by screening the Pfam database for proteins containing a catalytic PDD and one or more CBDs. The PDDs from this novel sequences and from known and biochemically validated CDAs were aligned with ClustalW, revealing predicted protein B2AAQ0, now known as *Podospora anserina* (S mat+) chitin deacetylase (PaCDA).

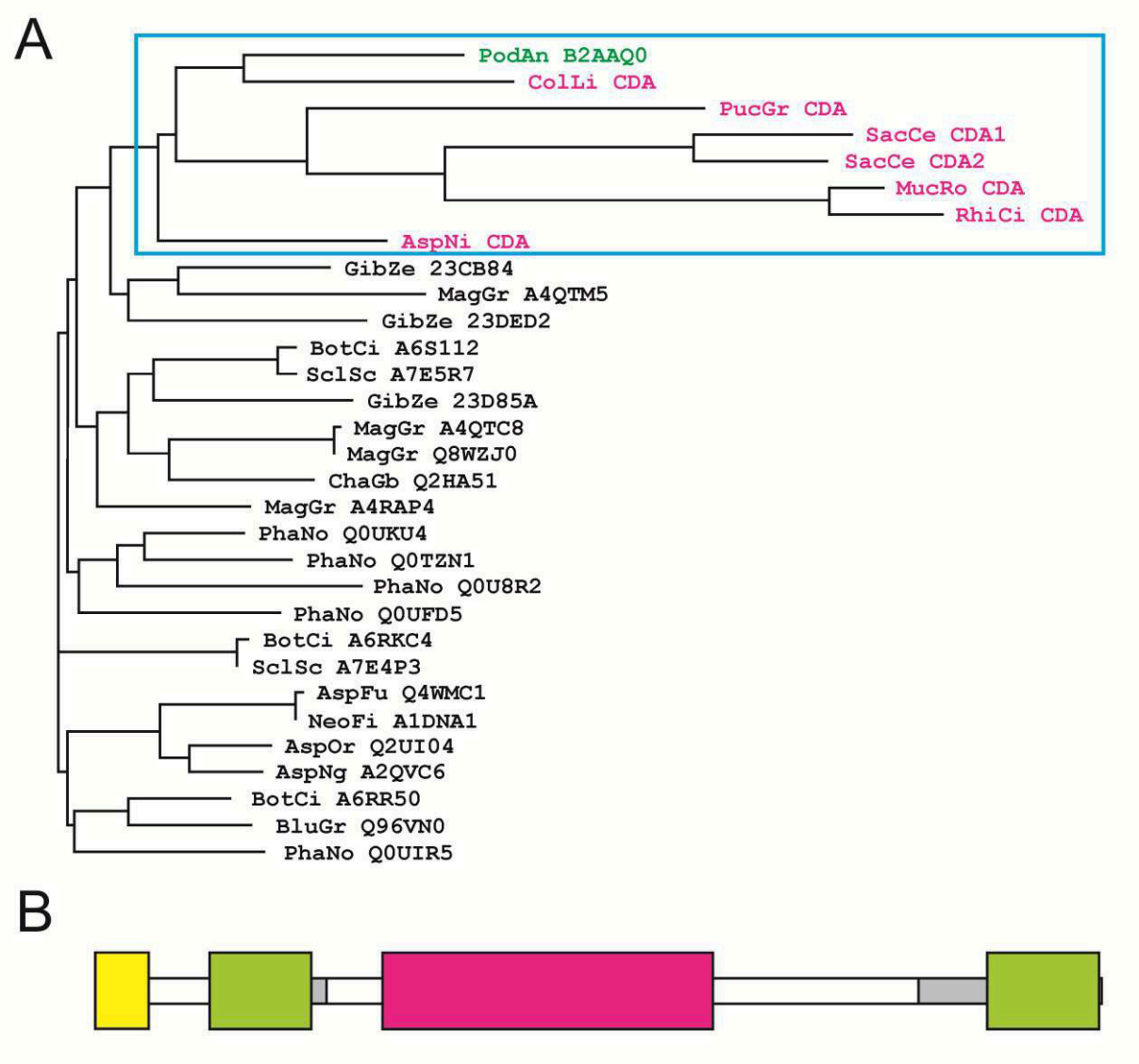

Fig. 1: Bioinformatic analysis: Cladogram (A) based on a protein sequence alignment (ClustalW2) of the polysaccharide deacetylase domains (PDD) of biochemically characterized CDAs (pink) and fungal sequences (black) containing additionally one or more chitin binding domains, *Podospora anserina* putative CDA B2AAQ0 (blue) is most closely related to the *Colletotrichum lindemuthianum* CDA (ColLi CDA) and the only sequence with CBD that is in the group of known CDAs (blue box), other species: *Puccinia graminis* f. sp. *tritici*, *Saccharomyces cerevisiae, Mucor rouxi, Rhizopus circinans, Aspergillus nidulans, Gibberella zeae, Magnaporthe grisea, Botrytis cinerea, Sclerotinia sclerotiorum, Chaetomium globosum, Phaeosphaeria nodorum, A. fumigatus, Neosartorya fischeri, A. oryzae, A. niger and Blumeria graminis.* **(B) Schematic representation of PaCDA** with 396 amino acids, green: chitin binding domain (PF00187), pink: polysaccharide deacetalase domain (PF01522), grey: linker region (low complexity), identified by Pfam, yellow: signal peptide, identified by SignalP.

This was the only protein that grouped with the known CDA sequences from *M. rouxi, R. circinans, S. cerevisiae* and *C. lindemuthianum* (Fig. 1A). The latter was the most closely related to PaCDA, both had 53% identity for the PDD sequences. PaCDA (Fig. 1B) is 396 amino acids in length, including a 21-residue N-terminal signal peptide identified by SignalP 3.0 (HMM result), as well as two CBDs located on both sides of the PDD, and contains no potential N-glycosylation sites predicted by NetNGlyc 1.0. The two CBDs were classified as CBM family 18 and differed in sequence and length. The fungal protein was estimated to feature ten disulfide bonds, four in each CBD and two in the PDD, thus making yeast the most sensible approach for heterologous production.

3.3.2. Production and purification of PaCDA

The PaCDA gene was optimized for extracellular expression in the yeast *H. polymorpha*, synthesized and appended with the sequence for a C-terminal StrepII-Tag. Multi-copy genome integration was successful as seen by pre-screening of the transformants for the presence of tagged protein. The second screening using glycol chitin dot gels confirmed the presence of correctly folded and active enzyme. Clones with CDA activity were cultivated on a larger scale (up to 1 L) and the recombinant enzyme was purified to homogeneity from the supernatant by StrepTactin affinity chromatography. Analysis of the recombinant protein using SDS-PAGE revealed a single band (Fig. 2A).

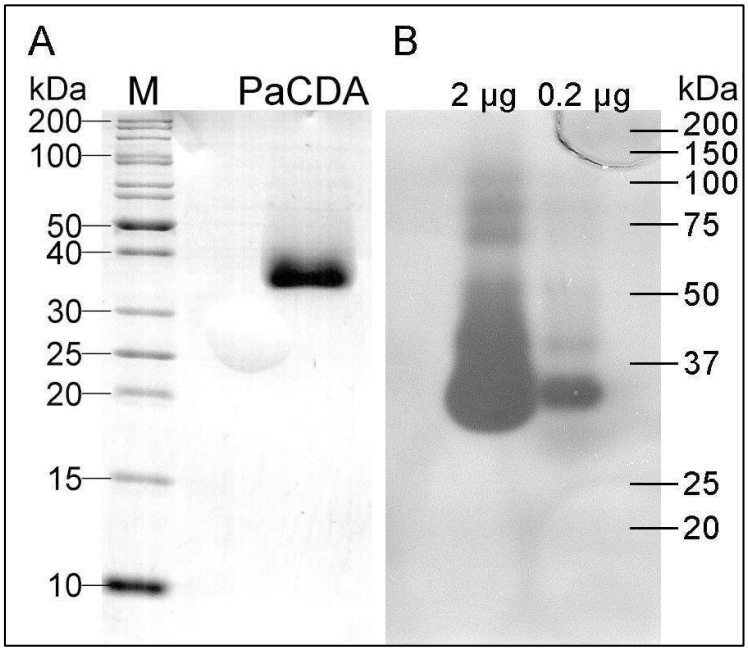

Fig. 2: Analysis of purified PaCDA, **A: SDS-PAGE** coomassie stained, M: peqGold Protein Marker II (unstained, Peqlab); **B: activity gel** after semi-native PAGE and incubation with overlay gel containing 0.1% glycol chitin, stained with Calcofluor white, after depolymerisation of formed chitosan by HNO_2, resulted in dark bands representing CDA activity, Marker: Precision Plus Protein Standards All Blue (Bio-Rad)

Although the predicted molecular weight of PaCDA is 40.5 kDa, it migrated as a 35.5 kDa band on the gel. The enzyme remained active following semi-native PAGE and deacetylated glycol chitin present in an overlay gel (Fig. 2B).

3.3.3. Substrates

Soluble glycol chitin, as used in the screenings and purification described above, is an artificial substrate. Polymeric chitosans with different DAs were deacetylated by the recombinant enzyme to different extents (Fig. 3A), although the same molar amount of acetyl groups was used. This clearly shows a preference for higher acetylated chitosans and suggests a corresponding subsite structure.

The activity of PaCDA against soluble chitin oligomers required a minimum DP of 2, but the activity compared to chitosan polymers was very low. Mass-spectrometric analysis showed that completely deacetylated oligomers were produced and that if an acetylated unit remained, it was mainly located at the reducing end of the pentamer and hexamer.

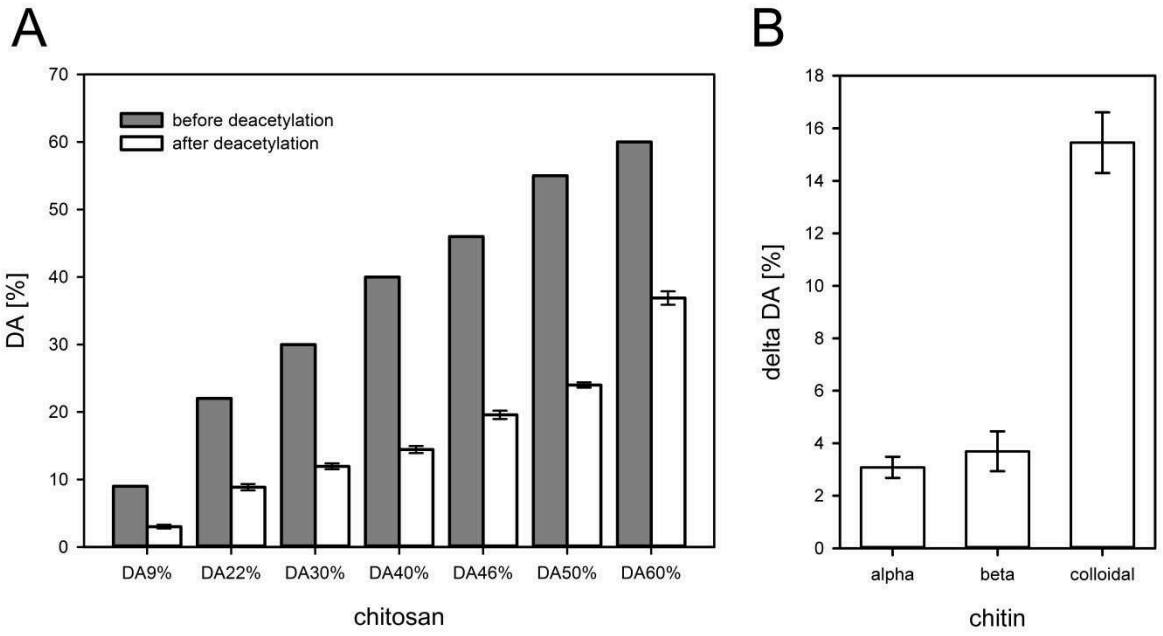

Fig. 3: PaCDA activity on chitosans (A) and chitins (B): chitosans with degree of acetylation (DA) ranging from 9% to 60% were incubated with 0.075 µM PaCDA at 37°C for 120 h, chitosan concentrations were adjusted to 0.05 g/l acetate groups, graph showing DA [%] before (grey) and after deacetylation by PaCDA (white). For the insoluble chitin substrates, the change in DA [%] is given, samples (1 g/l) were incubated with 0.246 µM PaCDA at 37°C and 180 rpm for 120 h.

Our main interest was focused on the ability of PaCDA to act on insoluble chitin polymers (Fig. 3B). Although there was low activity against α- and β-chitin, colloidal chitin was deacetylated by up to 15% and thus, to a substantially lower DA than achieved when other, known CDA had been used. We therefore used colloidal chitin for further enzyme characterization.

3.3.4. Characterization of PaCDA

The activity of PaCDA against colloidal chitin was also determined by measuring acetate release. The activity described an unusual non-Gaussian curve with a pH maximum at 10.5 (Fig. 4A) but was also stable over a wide pH range (5–8.5) so the optimal physiological pH can be considered to be ~8.5. We tested the temperature optimum at 1 h and pH 8.5 as shown in Fig. 4B, revealing a broad optimum of 40–60°C with a maximum at 55°C. Temperature stability testing at 80°C indicated a half-life of 11.8 ± 0.6 min.

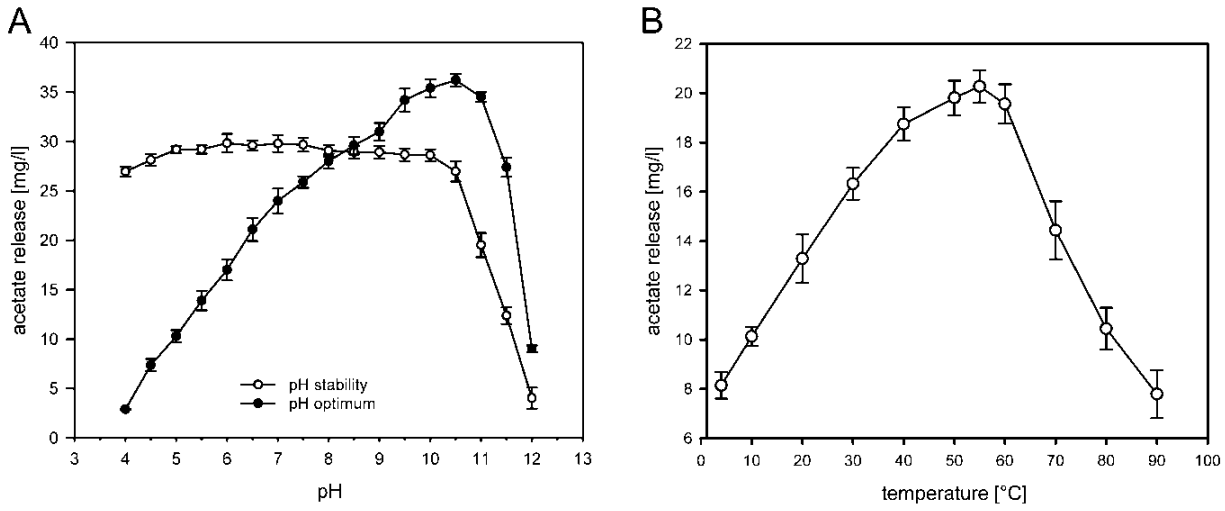

Fig. 4: Impact of pH and temperature, A: pH activity optimum and stability PaCDA (0,123 µM) incubated with 0.1% colloidal chitin at 37°C, for activity incubated 17 h in Teorell-Stenhagen-buffer pH 4 to 12, for stability pre-incubated 24 h in Teorell-Stenhagen-buffer pH 4 to 12, then the substrate was added and incubation 17 h in TEA pH 8.5 followed. **B: Temperature optimum:** activity of PaCDA (0,123 µM) on colloidal chitin (0.1%), samples incubated for 1 h at pH 8.5 (100 mM TEA, 100 mM NaCl), mg/l acetate release assayed for both experiments.

Neither EDTA nor β-mercaptoethanol inhibited the enzyme significantly unless combined with additional heating. The relative activity of the enzyme in the presence of these inhibitors and various bivalent cations is shown in Table 1. The values show that a tightly-bound cation is required for enzyme activity, but there is no requirement for a specific cofactor addition. Total reflection x-ray fluorescence (TXRF) analysis from the purified PaCDA showed that zinc is the most abundant element

among bivalent cations. The only other two detected elements were the known antagonists iron and copper. PaCDA activity was not inhibited by the presence of 100 mM acetate as well, tested by using chitin pentamer as substrate and analyzed by MALDI-TOF MS as described above.

Tab. 1: Activity of PaCDA with inhibitors (A) and **bivalent cations (B)**: relative activity [%] -/+ standard deviation on colloidal chitin, EDTA in mM, ß-mercaptoethanol in %

A: PaCDA – inhibitors and detaturation						
	H_20	**EDTA**	**β-merc.**	**β-merc./95°C**	**EDTA/ β-merc.**	**EDTA/ β-merc./95°C**
1 mM / 0.5%	100	99.6 ± 1.0	89.6 ± 4.6	67.9 ± 5.8	72.8 ± 1.4	1.1 ± 1.5
10 mM / 5%	100	60.7 ± 10.8	39.7 ± 0.5	0.5 ± 0.5	31.1 ± 1.7	-

B: PaCDA – bivalent cations							
	H_20	**Ca^{2+}**	**Mg^{2+}**	**Mn^{2+}**	**Fe^{2+}**	**Co^{2+}**	**Zn^{2+}**
1 mM	100	98.4 ± 2.1	98.7 ± 1.8	109.2 ± 2.1	102.8 ± 2.3	80.6 ± 5.5	31.7 ± 5.2
10 mM	100	98.0 ± 2.5	99.4 ± 2.7	77.4 ± 2.4	29.5 ± 5.7	19.1 ± 5.6	1.7 ± 1.7

3.3.5. Mode of action and pattern of acetylation

The mode of action of PaCDA was tested against different chitosan substrates. Two chemically-reacetylated chitosans were determined by ^{1}H-NMR to a DA of 61% and DA 33%, respectively. Chitosan with DA 61% was deacetylated by PaCDA to DA 29% (acetate release measured by coupled enzyme assay and verified by ^{1}H-NMR) and compared to solely-chemical chitosan of DA 33%. Fingerprinting was carried out using chitin/chitosan-degrading enzymes followed by MALDI-TOF MS analysis. The highly sequence-specific chitinosanase from *A. alternata* achieved the most distinct result. The two spectra in Fig. 5 show different sets of peaks. The chemical chitosan was degraded to 23 different chitosan oligomers varying in DP and DA. The PaCDA-treated chitosan resulted in a set of 15 peaks, lacking larger oligomers than DP 7. Although this measurement is not entirely quantitative, the oligomers containing two or more GlcNAc unit seem to be more abundant in the chemical chitosan spectrum, whereas the PaCDA products show higher peaks for oligomers with one GlcNAc unit up to the pentamer group.In addition to the MALDI-TOF MS the chitosans were analyzed by ^{13}C-NMR spectroscopy to determine the P_A value by calculating the diad frequencies of the C5 carbon atom.

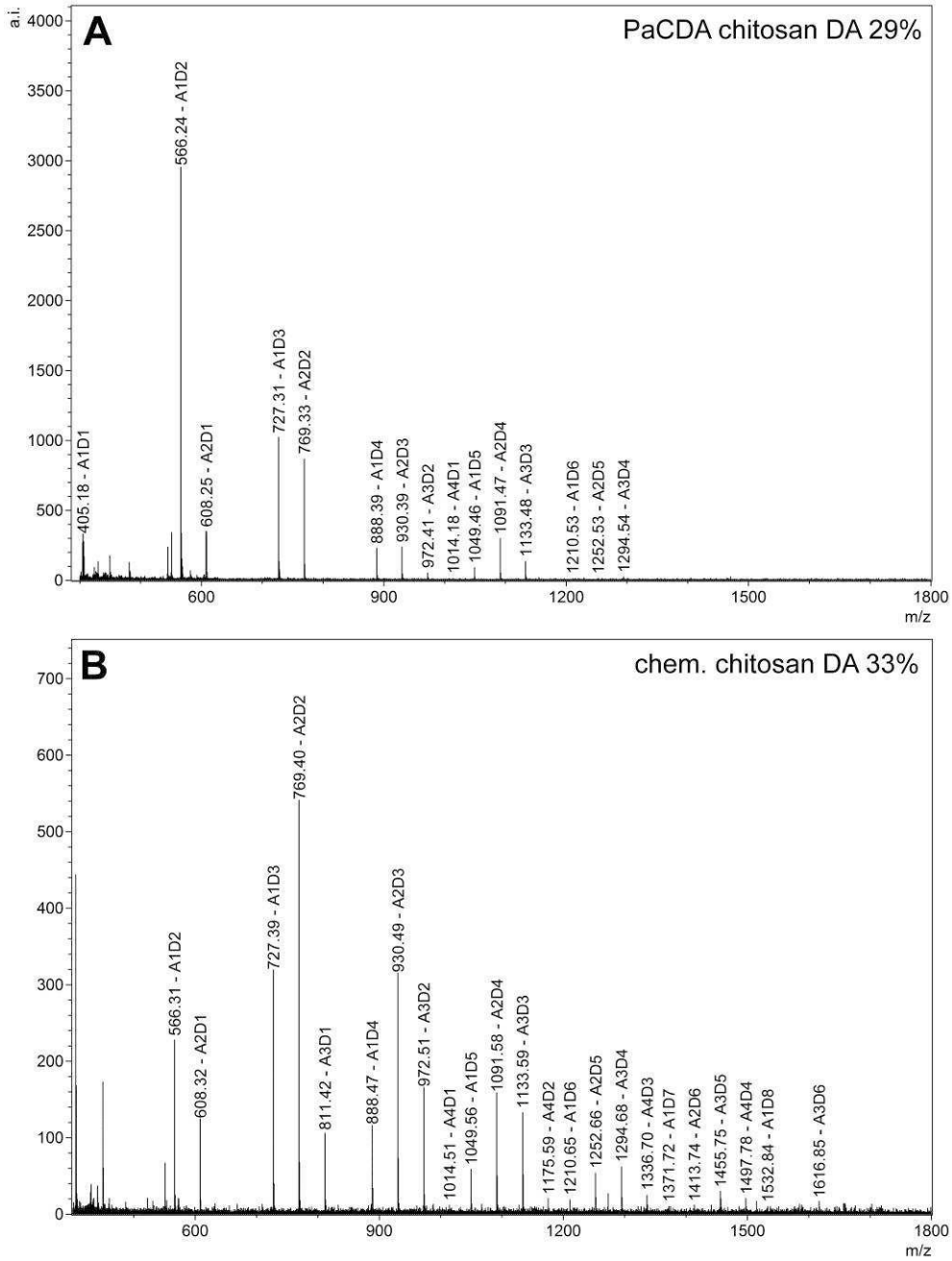

Fig. 5: Enzymatic/ mass spectrometric fingerprinting with MALDI-TOF MS: comparison of PaCDA deacetylated chitosan DA 29% (**A**) and chemically produced chitosan with DA 33% (**B**), samples hydrolyzed with sequence specific chitinosanase (*A. alternata*), resulting oligomers are annotated as A = GlcNAc, and D = GlcN unit.

The P_A of chemically-reacetylated chitosan DP 33% is 1.11, while the PaCDA-treated chitosan has a P_A of 1.33. By applying the equation 2 to a DP of 33%, the value for a random pattern of acetylation found by Weinhold et al. [2009] is 1.06.

Eq. 2

$$P_A = 1.11 - 0.58 \times e^{\left(\frac{-F_A}{0.13}\right)}$$

The chemically-reacetylated chitosan can therefore be regarded as random, whereas the value of the PaCDA-treated chitosan is differing and by definition on the side of a more alternating pattern. Part of the ^{13}C-NMR spectra at 600 MHz and 70°C showing the C5 resonance region is displayed in figure 6, where difference of the two chitosans can also be seen. The AA peak for example is much smaller in the spectrum of the PaCDA-treated chitosan and could not be quantified by the NMR evaluation software, which indicates GlcNAc units occur less often next to each other.

In summary of these two analytical methods the pattern of acetylation of enzymatically-derived chitosan is different to that of chitosan produced chemically.

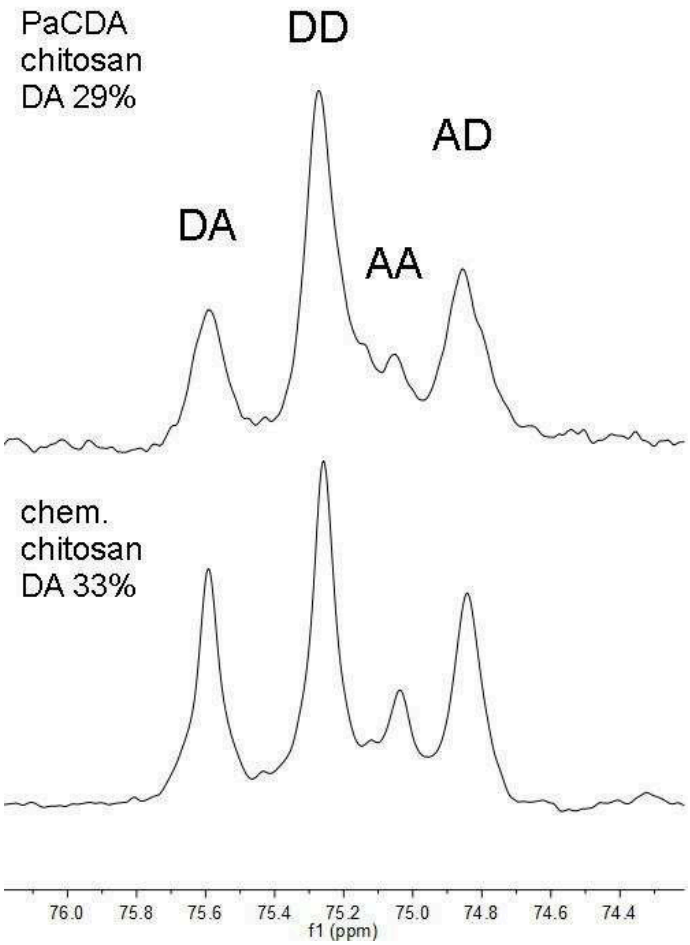

Fig. 6: PA analysis by ^{13}C-NMR: spectra recorded at 600 MHz and 70°C, excerpt of C5 carbon atom resonance region showing the diads DA, DD, AA and AD, (A = GlcNAc, D = GlcN), comparison of PaCDA deacetylated chitosan DA 29% and chemically produced chitosan with DA 33% reveals smaller AA peak.

3.3.6. Biological activities – Oxidative Burst assay

To test the biological activities of chitosans with different PAs, we chose the oxidative burst assay with *Oryza sativa* cell cultures. This assay measures elicitor-induced defense responses of plant cells by quantifying the reactive oxygen species (ROS) produced via the reaction with luminol derivate L012 in relative light units (RLU). At a concentration of 113 mg/l both chemical chitosans with a DA of 30% as well as the parent chitosan with a DA of 50% (as described above) were able to trigger an oxidative burst (Fig. 7). At first a PaCDA-treated chitosan tested did not elicit a strong oxidative burst like the two chemically produced (Fig. 7A), but the DA was later estimated to be only 18%. In the second experiment (Fig. 7B) chitosan with a DA of 30% was used and showed an oxidative burst curve almost as high as for the chemically-reacetylated chitosan with DA 30%, but with some lag-time. This and also in comparison to the DA 50% curve clearly indicates that for this assay the chitosans degree of acetylation is more important for the plant cell reaction that it's pattern.

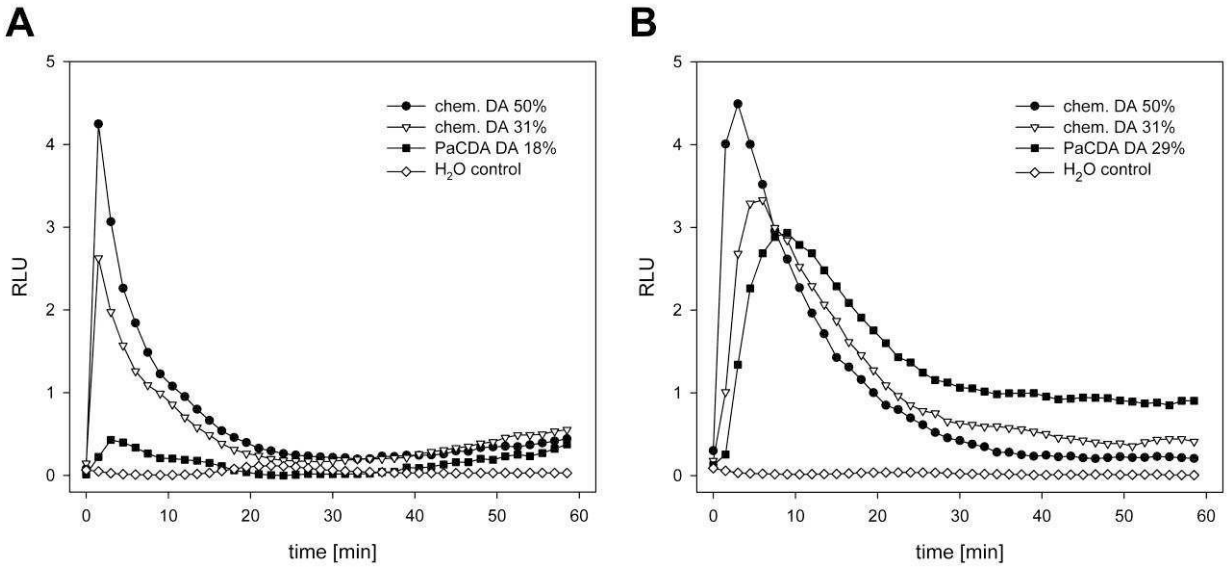

Fig. 7: Oxidative Burst assay: 113 mg/l of different chitosans were incubated with suspension cultured cells of *Oryza sativa* in 96-well microtiterplates, the production of ROS was measured as relative light units (RLU) after reaction with luminol derivative L012, mean of 5, representative of 7 or more experiments **A**: PaCDA deacetylated chitosan DA 18% was compared to chemically produced chitosans. **B**: showing same samples, except for PaCDA chitosan DA 30%.

3.4. Discussion

Chitosans can be produced from chitin either chemically or by enzymes (CDAs). These two processes are thought to generate chitosans that differ in terms of their patterns of acetylation (PA), which is in turn thought to influence their biological activities, although there has been no direct demonstration of this principle so far. Here we provide for the first time a chitosan with non-random pattern of acetylation produced by the novel enzyme PaCDA.

3.4.1. Bioinformatics

We searched for active CDAs containing CBDs because such enzymes have not been characterized before. This led to the identification of a novel CDA from *P. anserina*, containing one N-terminal and one C-terminal CBD (Fig. 1), with high similarity to ClCDA. Although genome databases contain multiple sequences like this, and some insect genes have been investigated [Dixit et al., 2008], this is the first CDA containing CBDs whose activity against chitinous substrates has been confirmed.

3.4.2. Production and purification of PaCDA

Our analysis revealed the presence of up to ten disulfide bonds in the protein, suggesting that production in *E. coli* would be challenging. We therefore selected as an alternative production host the methylotrophic yeast *H. polymorpha* [Hollenberg and Gellissen, 1997], also known as *Pichia angusta*, because this genus has been already been used to produce a CDA from *C. lindemuthianum* [Shrestha et al., 2004]. This is also the only fungal CDA whose crystal structure is known, and the investigation of this structure revealed an intermolecular interaction between the active site and His_6 tag [Blair et al., 2006]. To avoid this artifact we therefore chose the StrepII-Tag for one-step affinity purification. The purified protein migrated as a single band by SDS-PAGE (Fig 2A). The apparent molecular mass was lower than expected but this did not interfere with enzyme refolding or activity, as revealed by an in-gel assay against the substrate glycol chitin (Fig. 2B).

3.4.3. Substrates

Besides glycol chitin we carried out a series of in vitro assays against different substrates and found that PaCDA was not more active against the insoluble substrates α- and β-chitin than other CDAs [Martinou et al., 1995; Pareek et al., 2013], but was more active against colloidal chitin, which was deacetylated to a greater degree than achieved by *A. coerulea* and *C. lindemuthianum* CDAs [Win and

Stevens, 2001] or *Rhizopus oryzae* CDA [Aye et al., 2006]. This could reflect differences in the size of the chitin particles or the fine structure of the surface [Beaney et al., 2007]. However, we conclude that PaCDA worked only on the surface of crystals, but the CBDs may help the enzyme to attach to the substrate and thus increase its activity, which is a known effect [Bolam et al., 1998]. Originally identified as lectins, structural analysis of CBM famlily 18 containing proteins revealed they are made to bind small sugars (Type C), in contrast to the "surface-binding" (Type A) and "glycan-chain-binding" (Type B) structures [Boraston et al., 2004].

For chitin oligomers the minimum DP is 2, like for ClCDA [Tokuyasu et al., 1996] and *A. nidulans* CDA [Alfonso et al., 1995]. Due to the low activity the mode of action on oligomers was not studied further, but initial mass-spectrometric analysis showed it could be also very similar to ClCDA [Tokuyasu et al., 2000a]. Instead, chitosan DA 60% was chosen, which is a good substrate for PaCDA (Fig. 3A). The deacetylation of chitosans was carried out using the same molar amount of acetyl groups, therefore the different DAs afterwards suggest a specific PA is produced which is a poorer substrate for the enzyme.

3.4.4. Characterization of PaCDA

Although activity was tested against colloidal chitin and not glycol chitin, PaCDA behaves in a similar manner in terms of pH and temperature optimum as ClCDA [Tokuyasu et al., 1996; Tsigos and Bouriotis, 1995]. The shape of the pH curve can be explained as it is known that higher pH values favor deacetylation chemically, before the effect on enzyme stability dominates. Zn^{2+} appears to be the cofactor for PaCDA like is was shown previously for ClCDA [Blair et al., 2006]. This bivalent cation cannot be removed by EDTA and inactivation can only be achieved by denaturation with additional β-mercaptoethanol and heating, which adds to the compact structure of PaCDA. The thermo stability at 80°C is good; although the enzyme is not as heat-stable as *A. nidulans* CDA [Wang et al., 2009]. PaCDA was also unaffected by acetate, this finding groups the CDA again with the two enzymes mentioned before.

Considering the values compared to other CDAs known, PaCDA was highly similar to ClCDA not only in catalytic sequence but also in properties, and therefore most likely belongs to the group of endo-type CDAs which work non-processive and are supposed to have their biological role in plant–pathogen interactions [Tsigos et al., 2000].

3.4.5. Mode of action and pattern of acetylation

To study the mode of action, a "parent" chitosan (DP 1540 and DA 0%) was split into two samples, one of which was chemically reacetylated to 33% and one of which was chemically reacetylated to 61% and then enzymatically deacetylated to 29%, so that both samples had about the same DA but potentially differed PAs. This was investigated by enzymatic/mass spectrometric fingerprinting analysis with specific *A. alternate* chitinosanase; because this enzyme cleaves the polymer after a GlcN unit and a GlcNAc unit, the resulting oligomers must have GlcN-GlcNAc at the reducing end [Beneteau et al., in preparation]. The mass spectra for the two samples were distinct, with more oligomers containing only one GlcNAc unit for the PaCDA-treated chitosan. This indicates that PaCDA requires two side-by-side GlcNAc units for deacetylation, as also seen for the chitin oligomers. This is not evidence for a processive mode of action, but for substrate preference based on the PA. We therefore conclude that the PA of chitosans produced by PaCDA is not random but rather regular.

Analysis of chitosans by NMR spectroscopy for the distribution of units (=PA) was first described by Varum et al., [1990; 1991], who identified the diads and triads of GlcNAc (A) and GlcN (D) units. In case of PaCDA the ^{13}C-NMR analysis is even more convincing that the fingerprinting result. The spectrum of PaCDA-treated chitosan clearly shows a smaller peak for the AA diad, which supports the findings discussed before. The equation provided by Weinhold et al. [2009] using the diad frequencies of the C5 carbon atom to calculate the P_A value also allows numerical comparison. The P_A value of PaCDA-treated chitosan is not only different from the theoretical value for random distribution, but also from the value we determined for the solely-chemical chitosan with the same DP and DA. Martinou et al. [1998] studied a processive CDA from *M. rouxi* and found chitosans with slightly different diad frequencies from a Bernouillian distribution of units (=non-random PA) leading to the conclusion that the enzyme uses a multiple attack mechanism. In other words this is a more blockwise PA in comparison to the more regular pattern we have found for the PaCDA. The hypothesis that enzymes can/should/must make a non-random pattern of acetylation was suggested some time ago [Tsigos et al., 2000; Zhao et al., 2010], now we not only have shown it is feasible to produce non-random PA chitosans with a non-processive enzyme, but also that the resulting PA is different using another type of CDA. Generally, enzymes have structurally-specific substrate preferences which in the case of CDAs would promote the creation of chitosans with non-random blocks of PAs based on the individual preferences at each subsite.

3.4.6. Biological activities

As fungal CDAs are known to play a role in plant-pathogen interactions [El Gueddari et al., 2002] and chitosans are biologically active in plant systems [Hadwiger, 2013], it was obvious to test enzymatically derived chitosan as well. A measurable indicator of plant defense mechanisms are reactive oxygen species (ROS), in particular H_2O_2. The oxidative burst assay quantifies the reaction towards elicitor molecules and it is known that chitin hexamer and chitosan polymers can trigger H_2O_2 production [Ortmann et al., 2004; Paulert et al., 2010]. We directly compared different chitosan polymers and found that higher DAs resulted in higher peaks, but we could not see a clear difference between PaCDA-treated and chemically-reacetylated chitosan with DA 30%. It already has been shown that the DP of chitosan has an influence on H_2O_2 production [Liu and He, 2011] and so has the DA on necrosis in wheat leaves [Vander et al., 1998]. Here we confirm that the latter is also the case for oxidative burst in rice suspension cultures, as well taking into consideration that *O. sativa* chitin elicitor binding protein (CEBiP) includes two chitin binding motives (LysM), which recognize the GlcNAc units [Kaku et al., 2006]. Nevertheless it is possible that the PA matters to the plant cells and makes a difference for biological activity. We might speculate that isolated GlcNAc units as present in a regular patterned chitosan are not efficient in eliciting resistance reactions. We may further speculate, based on previous results with other weak elicitors [Ortmann et al., 2006], that these chitosans may be used to prime the cells to prepare them for defense. Therefore oxidative burst assays for priming have to be carried out in the next step. If these can confirm the speculations, this is a promising result for chitosan applications in the field of plant protection.

3.5. Conclusions

PaCDA is able to work on different substrates and although it has two CBDs is not able to break up insoluble chitins, therefore the role of CBDs on enzyme properties and mode of action have to be investigated. In PDD sequence and enzyme properties PaCDA is very similar to ClCDA, but it is producing a chitosan with a specific, non-random pattern of acetylation, which was confirmed by fingerprinting and NMR analysis. Because the oxidative burst assay did not show differences towards chemically derived, random chitosan other biological assays (e.g. for anti-microbial activity) have to be carried out.

The ability to generate non-random PAs by using PaCDA and other enzymes creates the feasibility to tailor bioactive properties for applications in plant strengthening, biological control of fungal pathogens and even medical applications.

Chapter 4

Analysis of chitin binding domain deletion variants of *Podospora anserina* chitin deacetylase

Abstract

Chitosan is a natural carbohydrate product with a wide range of applications depending on its properties like the degrees of polymerization and acetylation, both influencing its biological activities. It was recently demonstrated that a chitin deacetylase from *Podospora anserina* (PaCDA) is capable of generating chitosans with a non-random pattern of acetylation (PA), which are clearly different from random PA chitosans that can be produce chemically, and therefore offer new possibilities for application. PaCDA is very similar to the well characterized *Colletotrichum lindemuthianum* CDA in protein sequence of the catalytic domain and enzyme characteristics, but differs in the domain structure by carrying two additional chitin binding domains (CBDs).

In the present study the role of the CBDs for PaCDA was investigated by constructing deletion variants lacking the N-teminal (ΔN), the C-terminal (ΔC) and both domains (ΔNC). As for the full-length PaCDA, production of the truncated enzymes was carried out using *Hansenula polymorpha*. However, this was not successful for PaCDA-ΔC and ΔNC indicating that the C-terminal deletion caused incorrect protein folding. Additionally, CDA activity was discovered in control samples of expression host *H. polymorpha* strain without any PaCDA gene. Due to these challenges, production of PaCDA in *E. coli* was tested, but proved to be no alternative in comparison to the yeast system. Nevertheless, PaCDA-ΔN could be produced and purified, followed by the comparison with full-length PaCDA in terms of pH and temperature optimum as well as the mode of action on chitosan polymers. While showing the same activity on colloidal chitin, PaCDA-ΔN favored lower temperatures and led to slight differences in PA analyzed by enzymatic/mass-spectrometric fingerprinting.

4.1. Introduction

Chitin deacetylase from *Podospora anserina* (PaCDA) has been heterologously produced in *Hansenula polymorpha* and characterized as reported in the previous chapter. The enzyme is able to generate chitosan with non-random pattern of acetylation (PA) and deacetylates the insoluble colloidal chitin to an extent of 15%. Using this substrate the optimal pH was 10.5, the temperature optimum was 55°C and Zn^{2+} was found as a cofactor. These and other values were similar to those observed for the *Colletotrichum lindemuthianum* CDA (ClCDA) [Tokuyasu et al., 1996; Tsigos and Bouriotis, 1995], to which PaCDA also has the highest sequence similarity concerning the polysaccharide deacetylase domain (PDD). The particularity of PaCDA lies in the additional two chitin binding domains (CBDs), one of them being located N-terminally to the PDD and the other one at the end of the C-terminus. As already mentioned in the chapters describing the CDAs from *Botrytis cinerea* and *P. anserina*, binding modules occur in carbohydrate degrading enzymes or individually in binding proteins [Boraston et al., 2004]. As the activity of the *B. cinerea* putative CDA BC95 could not be demonstrated, PaCDA is the first active CDA with CBDs reported.

Coding sequences of CBDs have been deleted from bacterial [Hashimoto et al., 2000] and plant [Iseli et al., 1993] chitinase genes and the truncated enzymes had lower activity towards β-chitin microcrystals or decreased affinity to colloidal chitin, respectively. The other way around, addition of binding domains to a chitinase resulted in stronger binding and higher activity on insoluble, but not to soluble substrates [Limón et al., 2001]. Thus for PaCDA the question arose, if the CBDs may influence the substrate specificity, especially to insoluble substrates like colloidal chitin, as well as the mode of action and the resulting PA of chitosan. The role of the CBDs for the PaCDA enzyme was the subject of the master's thesis of Nils Gilleßen ["Functional characterization of chitin binding domains in *Podospora anserina* chitin deacetylase", 2012], who was supervised by me. His work describes part of the screening for production clones, the production and purification of the three truncated variations and their comparison in enzymatic means. In this chapter, I will present only a short summary of these results and additionally cover the generation of the constructs lacking one or both CBDs, the initial screenings and enzymatic/mass-spectrometric fingerprinting of enzyme products. In the end, I added results from experiments to analyze the possibility to use *E. coli* as an alternative expression host for PaCDA and variations. The discussion will present all findings regarding the PaCDA deletion variants, including the challenges during production and purification.

4.2. Materials and Methods

4.2.1. Generation of PaCDA deletion constructs

An alignment of the protein sequences of PaCDA and ClCDA using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2) was the basis for constructing truncated versions of PaCDA (Fig. 1). The deletion of CBDs was achieved by a PCR-based strategy using the plasmid coding for the PaCDA gene pMA-T::PaCDA-StrepII as template. For amplification, back-to-back phosphorylated primers 5'-pho-TGGTCTCATCCTCAATTTGAAAAG-3' and 5'-pho-AGCGTCTCTGTACCAGTTAAC-3' were used for PaCDA-ΔN and 5'-pho-TCTACCCCAAGACCAAAGTTC-3' and 5'-pho-ACTGAGAGT TAGCGTCTCTC-3' for PaCDA-ΔC. Location of the deletions within the PaCDA sequence is shown in Fig. 1 and indicated by black arrows in Fig.2. The PCR was carried out using Phusion™ Hot-Start High-Fidelity DNA Polymerase (Finnzymes, Thermo Scientific). This was followed by digestion of the amplicons obtained with *Dpn*I. Afterwards the PCR products were extracted from agarose gels and self-ligated. To construct the double deletion PaCDA-ΔNC, this procedure was repeated with the single deletion plasmids, each with the other primer pair. The plasmids obtained were all checked by sequencing and sent to Artes Biotechnology GmbH (Langenfeld, Germany), where the three coding sequences of PaCDA-ΔN, PaCDA-ΔC and PaCDA-ΔNC were cloned in expression vector pFPMT121. Production strains were generated equal to the full-length PaCDA strain (chapter 3.2.2); including the pre-propeptide MF-α1 coding sequence for extracellular production in *H. polymorpha* host strain RB11. Transformants underwent the procedure of passaging and stabilization for mitotically stable integration of multiple copies into the yeast genome. A pre-screening by western blot and was carried out by Artes to identify the best protein producing pools of yeast clones.

4.2.2. Screening, production and purification of PaCDA deletion variants

Stabilized *H. polymorpha* transformants were cultivated exactly like the full-length PaCDA strain on YPD agar for strain maintenance and in YPYNBG medium for de-repression, using 3 ml in tubes for screening and 200 ml each in several 2-l flasks for production cultures up to 1.2 l in total. I screened pools of *H. polymorpha* clones for active enzyme in undiluted culture supernatant, soluble and insoluble cell extract samples. The protocol for *H. polymorpha* cell disruption can be found in chapter 2.2.5.8, other samples were prepared and analyzed as described in the materials and methods section of the PaCDA manuscript (chapter 3.2.), where the details of all following methods can be found as

well. Later, clones of all deletion constructs were individualized and screened again for highest activity of culture supernatant dilutions 1:1, 1:10, 1:100 and 1:1000. The CDA activity screening was carried out by an assay using 0.1% glycol chitin gels with 1-µl samples applied as drops and activity was visualized after Calcofluor white staining and chitosan depolymerization [Trudel and Asselin, 1990]. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and western blotting with Strep-Tactin® HRP conjugate (IBA, Göttingen, Germany) followed by enhanced chemiluminescence (ECL) detection was also carried out accordingly. For the purification of the Strep-tagged proteins a 1-ml Strep-Tactin Superflow Plus column (Qiagen) was used for FPLC in an ÄKTAprime plus system (GE) following the manufacturers' recommendations. Additionally, Gilleßen investigated the purification of PaCDA-ΔC and PaCDA-ΔNC also by anion and cation exchange chromatography and carried out a large-scale cell disruption for the usage of the intracellular protein fraction [Gilleßen, 2012].

4.2.3. Comparative characterization of PaCDA and PaCDA-ΔN

The direct comparison of PaCDA and PaCDA-ΔN in terms of pH and temperature optimum was done by Nils Gilleßen in the context of his master's thesis in 2012 using my protocols for PaCDA. Accordingly, colloidal chitin (0.1 g/l) was used as substrate, 25 mM Teorell-Stenhagen buffer for the pH range from 3-12 and temperatures between 4°C and 90°C were assayed with enzyme concentrations adjusted for the same molarity. Samples were incubated at 37°C and 750 rpm for 15 h and 1 h, respectively. The enzyme activity was measured by quantifying the acetate release by threefold coupled enzyme assay [Martinou et al., 1995] using solutions of an acetic acid assay kit (R-Biopharm AG, Darmstadt, Germany) in 96-well mircotiterplates. The preparation of all buffers and substrates used, including the chitosan DA 50%, are described in the PaCDA manuscript (chapter 3.2).

4.2.4. Enzymatic / mass-spectrometric fingerprinting of CDA products

Other than in the PaCDA manuscript, chitosan DA 50% (0.5 g/l) was used for this fingerprinting analysis and deacetylated by 0.3 µM PaCDA-ΔN and full-length enzyme in 1-ml samples of 50 mM volatile ammonium carbonate buffer for 120 h. The resulting DA was calculated after the measurement of acetate released. The hydrolysis of deacetylated chitosans for analysis by the chitinosanase from *Alternaria alternata* and the subsequent Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) were carried out as described in the previous chapter 3.2.6.

Samples were analyzed using 2,5-dihydroxybenzoic acid (DHB) matrix solution, 355 nm SmartBeam™ NdYAG-laser and Bruker Daltonics Autoflex Speed (Bremen, Germany) followed by evaluating the spectra with Mmass [Strohalm et al., 2010] and annotating the composition of found oligomers as GlcNAc (A) and GlcN (D) residues.

4.2.5. PaCDA production in *E. coli*

A derivative of the pET-22b(+) vector (Novagen, Merck KGaA, Darmstadt, Germany) including a StrepII-tag coding sequence downstream of the multiple cloning site was used for the heterologous production of PaCDA in *E. coli.* The PaCDA coding sequence was amplified using pMA-T::PaCDA-StrepII as the template, Phusion™ Hot-Start High-Fidelity DNA Polymerase (Finnzymes) and primers 5'-GGAATTGCATATGGACGCTAACTCTCAGTCTACTTCG-3' and 5'-CGAGCTCGTTACAGTTG CCGAAGGCTCTC-3' (*Nde*I and *Sac*I restriction sites used for cloning are underlined). The Twin-Strep-tag was introduced by the two complementary primers 5'-CGAGCTCAGTGCTTGG TCTCATCCTCAATTCGAGAAAGGAGGCGGTTCTGGAGGCGGTTCAGGAGGCAGCGCTACTCGAG CGG-3' and 5'-CCGCTCGAGCGCTGCCTCCTGAACCGCCTCCAGAACCGCCTCCTTTCTCGAA TTGAGGATGAGACCAAGCACTGAGCTCG-3' that were annealed by lowering the temperature gradually from 98°C to 60°C. The DNA fragment obtained was subsequently digested by *Sac*I and *Xho*I and inserted into the *Sac*I/*Sal*I-cut plasmid pET-22b(+)::PaCDA-StrepII.

The production strains *E. coli* Rosetta 2 (DE3) [pLysS-RARE2] and *E. coli* Rosetta 2 (DE3) [pLysS-RARE2, pCDFDuet1::DSBC/TrxA] were transformed via heat shock with the plasmid pET-22b(+)::PaCDA-StrepII. While the plasmid pLysRARE2 carries the sequences for stress-induced autolysis, chloramphenicol resistance and rare codons, pCDFDuet1::DSBC/TrxA, based on the pCDF Duet™ vector (Novagen), includes a streptomycin resistance cassette and two genes: one coding for a prokaryotic disulfidebridge isomerase (DsbC) with chaperon activity and one for thioredoxin A for a reducing environment [de Marco, 2009].

For protein production, transformed strains were cultivated in auto induction medium [Studier, 2005] including the appropriate antibiotics, as described in the *Botrytis cinerea* CDA manuscript (chapter 2.2.5.6). Cell lysis, protein purification and analytics were also carried out using protocols, which can be found in the previous chapter 2.2.5.

4.3. Results

4.3.1 Bioinformatics and cloning

The PaCDA gene was chosen for heterologous expression because of its high similarity to the ClCDA gene in the PDD coding sequence, as stated in the chapter before. An alignment of the protein sequences of PaCDA and ClCDA is pictured in figure 1, where it shows that the number of identical amino acids is high in the PDD region as well as in the entire C-terminus of ClCDA. Thereafter ClCDA is not only lacking the C-terminal CBD, but also the complete low complexity region, hence both were deleted for the PaCDA-ΔC variant. I proceeded accordingly with the N-terminal CBD, were also the CBD sequence and additional amino acids were deleted to connect both remaining parts with a serine followed by a threonine.

```
PaCDA           MINVPVTLLSLSAVATAAALADANSQSTSPNPSPRQHARDTLQKRANCGAGIGNCPSGQC 60
ClCDA           -----MHFSTLFGAAATAALAG------STNASP-------LARRQ-------------- 28
                     : : :* ..*::****.      *.*.**       * :*

PaCDA           CSQYGWCGVTSEHCGTGCQSGFGTCTGGGGGNNEETLSTPRPKFGSIPYGVTITNCNAAG 120
ClCDA           ----------------------------------------VPVGTPILQCTQPG 42
                                                        :* *..* :*. .*

PaCDA           TIALTFDDGPFLYTNQLLDLLAQQQVKATFFTNGLNWGDATQAPYPDVLRRIVNDGHQLG 180
ClCDA           LVALTYDDGPFTFTPQLLDILKQNDVRATFFVNGNNWANIEAGSNPDTIRRMRADGHLVG 102
                 :***:***** :* ****:* *::*:****.** **.:   .. **.:**:  *** :*

PaCDA           SHTYNHPDLNTLTTAARRSNMAQNEKIFKDALGGYFPTYMRPPYGSCTGQCLTDLGDLGY 240
ClCDA           SHTYAHPDLNTLSSADRISQMRHVEEATR-RIDGFAPKYMRAPYLSCDAGCQGDLGGLGY 161
                **** *******::* * *:* : *:  :  :.*: *.***.** ** . *  ***.***

PaCDA           HVINWNIDTLDYQGNIPN----SQNIFNSAVSTNAAANKYIALAHDVHQATVQQLALGLI 296
ClCDA           HIIDTNLDTKDYENNKPETTHLSAEKFNNELSADVGANSYIVLSHDVHEQTVVSLTQKLI 221
                *:*: *:** **:.* *:    * : **. :*::..**.**.*:****: ** .*:  **

PaCDA           QTAKNRGYRLVTVGECLGDAPVNWYRDATTGNARTGGGGGSGGNPNPGPITSTNGLCGST 356
ClCDA           DTLKSKGYRAVTVGECLGDAPENWYKA---------------------------------- 248
                :* *.:*** ********** ***:

PaCDA           NGNMNCLNSGFGNCCSQWGFCGSTAEYCGANCQRAFGNCN 396
ClCDA           ----------------------------------------
```

Fig. 1: Protein sequence alignment of *Podospora anserina* and *Colletotrichum lindemuthianum* chitin deacetylases (PaCDA and ClCDA) by ClustalW 2.1, used for construction of PaCDA ΔCBD variants, pink: polysaccharide deacetylase domains, green: chitin binding domains, grey: low complexity regions (identified by Pfam), yellow: signal peptides (idendified by SignalP), deleted regions underlined.

First the single deletion constructs PaCDA-ΔC and PaCDA-ΔN were generated and used for the construction of PaCDA-ΔNC. A schematic illustration of the full length protein and the truncated variants is shown in figure 2, were the position of the primers used is also indicated. Like PaCDA before, the three mutated gene were cloned for heterologous expression in *H. polymorpha* by Artes Biotechnology GmbH, were the corresponding production strains were developed.

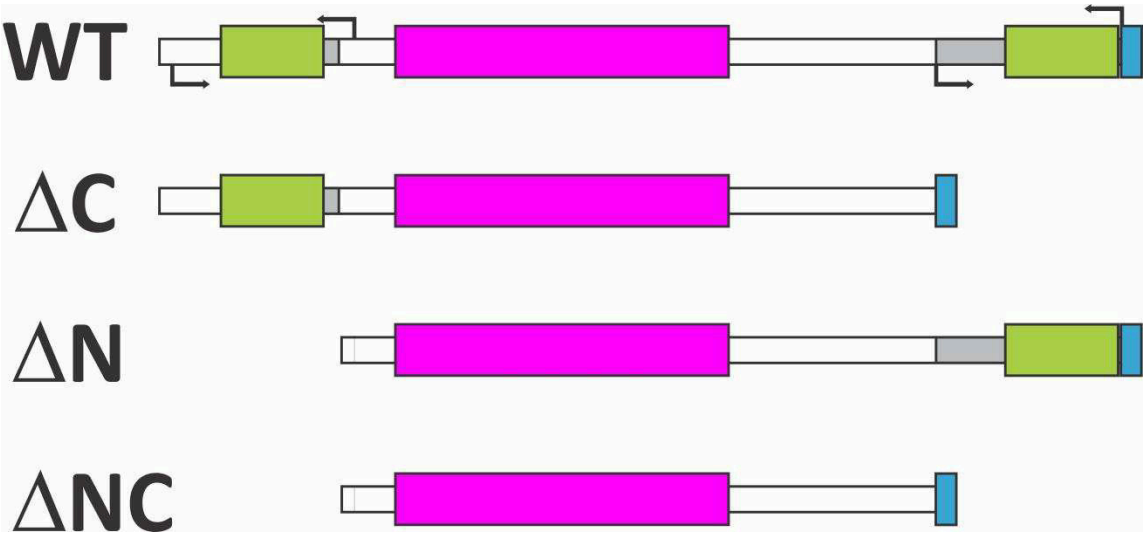

Fig. 2: Schematic structure of PaCDA and truncated variants with lacking C-terminal (ΔC), N-terminal (ΔN) or both (ΔNC) chitin binding domains (green), polysaccharide deacetylase domain (pink), low complexity regions (grey) and StrepII-tag (blue), arrows indicate deletions.

4.3.2. Screening of transformants

Pools of transformants were pre-screened by Artes on dot blots with culture supernatants and Strep-Tactin HRP conjugate detection, in order to identify the best producers of the PaCDA deletion muteins PaCDA-ΔN, PaCDA-ΔC and PaCDA-ΔNC. Two pools each were selected for ΔN (#9 and #16) and ΔNC (#9 and #12) and three for ΔC (#2, #4 and #18) and analyzed by western blotting of extracellular, soluble and insoluble intracellular fractions. As already seen on the dot blot, PaCDA-ΔN (33.4 kDa) was secreted into the culture supernatant, but western blot confirmed that weak signals seen for PaCDA-ΔC (33.6 kDa) and PaCDA-ΔNC (26.5 kDa) were due to a poor secretion. Both proteins were mainly found in the insoluble cell fraction, thus the production of correctly folded and processed protein was questioned.

All seven pools of clones were tested for CDA activity on dot gels with 0.1% glycol chitin (Fig. 3) after I received the *H. polymorpha* cultures. CDA activity could be detected as dark spots after depolymerization of chitosan in all pools and fractions; thereby the spots of the two ΔN samples were as big as the full-length PaCDA. PaCDA-ΔC and ΔNC showed clearly lower activity, being the weakest in the PaCDA-ΔNC supernatants. A small amount of chitinase activity, represented by dark spots before depolymerization, was present in the supernatants as well as in the insoluble protein fractions of all pools, but mostly seen in the Mock samples.

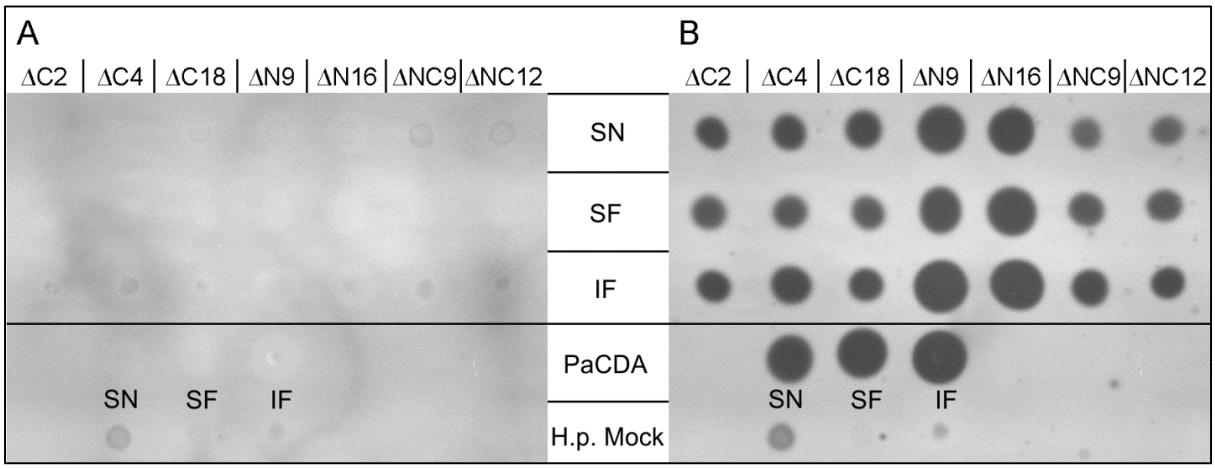

Fig. 3: CDA activity screening of PaCDA deletion constructs using 0.1% glycol chitin dot gel assay stained with Calcoflour white, before (**A**) and after depolymerisation of chitosan (**B**), pools (numbers) of variants lacking C-terminal (ΔC), N-terminal (ΔN) or both (ΔNC) chitin binding domains analyzed for CDA activity in culture supernatant (SN), soluble (SF) and insoluble fraction (IS) of cell extracts, PaCDA wild type and *H. polymorpha* Mock strain as controls.

Simultaneously the three fractions were analyzed by western blotting and the results confirmed the findings previously obtained by Artes (Fig. 4). Clear bands of secreted protein can only be seen for full-length PaCDA and the two ΔN pools, whereas it is missing in the supernatants of ΔC and ΔNC pools. The insoluble intracellular fractions of PaCDA-ΔC and ΔNC contain many detected proteins of different sizes forming different band patterns for each variant. These represent most probably aggregation and degradation products of the tagged PaCDA variants, because no signals are visible in the lanes of the mock negative control. The full-length PaCDA with 40.5 kDa acts in SDS-PAGE like a 35.5-kDa protein, and the PaCDA-ΔN (33.4 kDa) appears at 30 kDa, thus the other found bands could not definitely be assigned to the truncated proteins. Due to the CDA activity present in the samples the screening was carried on with clones of all variations.

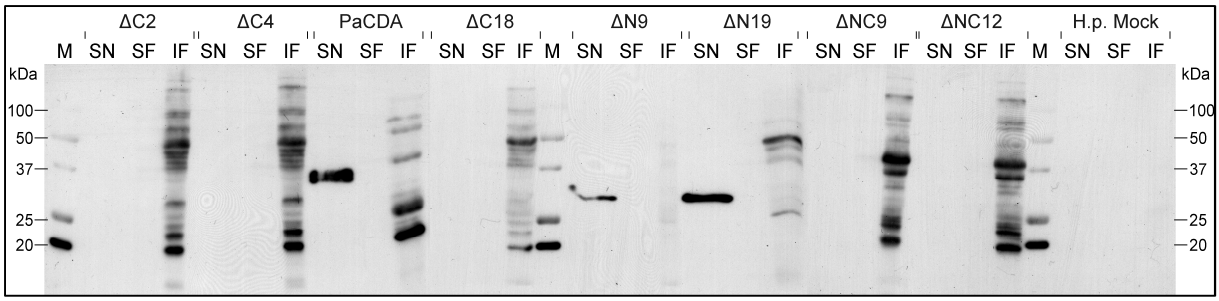

Fig. 4: Western blot analysis of PaCDA deletion constructs: pools (numbers) of variants lacking C-terminal (ΔC), N-terminal (ΔN) or both (ΔNC) chitin binding domains analyzed for presence of StrepII-tagged protein in culture supernatant (SN), soluble (SF) and insoluble fraction (IS) of cell extracts, PaCDA wild type and *H. polymorpha* Mock strain as controls, marker: Precision Plus Protein All Blue Standards (Bio-Rad).

Clones of all pools were individualized and screened for the best producer of active enzyme by spotting dilution series of culture supernatants onto the substrate gels. Individual clones of PaCDA-ΔN9 and ΔN16 showed similar activity levels, delectable even with 1:1000 dilutions, thus clones ΔN9-F and ΔN16-B were selected for production. Analogously the screening of pools PaCDA-ΔC18 and ΔNC12 was carried out by Nils Gilleßen and clones ΔC18-A and ΔNC12-A were chosen.

4.3.3. Production and purification

These experiments were carried out and reported in detail by Gilleßen as well. Clone PaCDA-ΔN16-B was cultivated for enzyme production like described for the full-length PaCDA and PaCDA-ΔN was purified from the culture supernatant via Strep-Tactin affinity chromatography using the same protocol like for PaCDA as well. To analyze the purity, SDS-PAGE and western blot were carried out including PaCDA and using 2.5 µg protein samples, which showed a clear band at 30 kDa and no contaminants for PaCDA-ΔN (Fig. 5A). Immunodetection with Strep-Tactin HRP conjugate after western blotting identified the band as recombinant protein (data not shown).

Affinity chromatography was also applied to PaCDA-ΔC and PaCDA-ΔNC with extracellular and intracellular protein fractions, but failed for both variants as well as for both sources. A glycol chitin gel with all FPLC fractions showed the highest CDA activity in the flow through, pointing out that the protein did not bind to the column as expected.

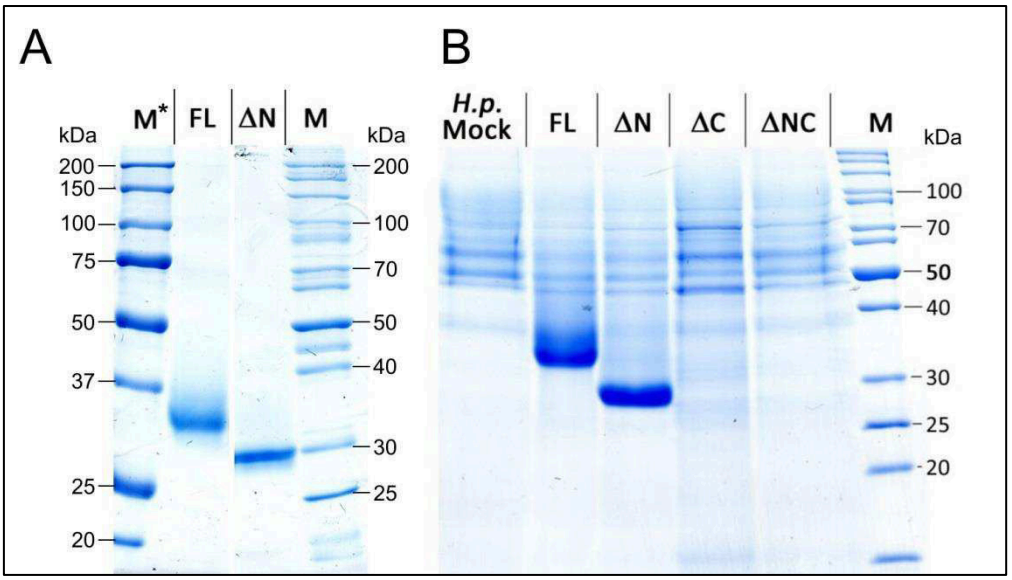

Fig. 5: SDS-PAGE of PaCDA and variants, Coomassie staining, M*: Precision Plus Protein Standards All Blue (Bio-Rad), M: peqGold Protein Marker II (Peqlab) **A: PaCDA and PaCDA-ΔN** purified protein 2.5 µg each, **B: all variants** including negative control (Mock), 12 µg culture supernatants each [modified from Gilleßen, 2012]

Heterologously expressed PaCDA is known to be the major protein in *H. polymorpha* culture supernatants; therefore other purification methods seemed accomplishable. Gilleßen decided in favor of anion exchange chromatography, because of the theoretical isoelectric points (pI) of 6.3 and 6.1 for PaCDA-ΔC and PaCDA-ΔNC, respectively. As seen before, enzyme activity was also ending up in the flow through. A likely explanation for this is that the actual pI can be 8 or higher, which makes cationic exchange chromatography a method to choose. The attempts with this method did neither yield purified enzymes.

As the PaCDA variants ΔC and ΔNC could not be purified with any of the described methods, the production of heterologous protein was monitored in detail. Therefore the different versions were compared on SDS-PAGE, western blot as well as in CDA activity assays. On the Coomassie stained gel (Fig. 5B) there are some weak bands around 30 kDa visible for PaCDA-ΔC and ΔNC, that are not present in the control, but no clear “overexpression bands” (heterologously produced protein bands) like for PaCDA and ΔN. The Western Blot showed strong StrepII-Tag signals for full length and ΔN enzyme and no signal at all for the other two truncated proteins (data not shown). These results let to the conclusion that only very little amounts of PaCDA-ΔC and ΔNC are secreted and that the StrepII-Tag is somehow not accessible. The CDA activity assay with glycol chitin gel was carried out like before, but revealed that some CDA activity seems to be also present in the supernatant of the

H. polymorpha control strain (Fig. 6). While the chitinase activity is known, the CDA activity has never been seen before, as the spot of the undiluted sample is clearly darker after depolymerization. Questioning the findings described before regarding the activity of ΔC and ΔNC proteins, another activity assay was followed up, which is independent from any chitinase activity. The culture supernatants adjusted for protein content were incubated with colloidal chitin and activity was measured following acetate release like described detailed in the PaCDA chapter. This test confirmed the same activity level for PaCDA and ΔN enzyme and quantified the activity of PaCDA-ΔC at about the half of the foresaid. Regarding the PaCDA-ΔNC variant, there was less acetate measured, as for the control strain without recombinant protein. In summary, the presence of active CDA in the unmodified *H. polymorpha* strain was proven and the activity of the double deletion was not detected.

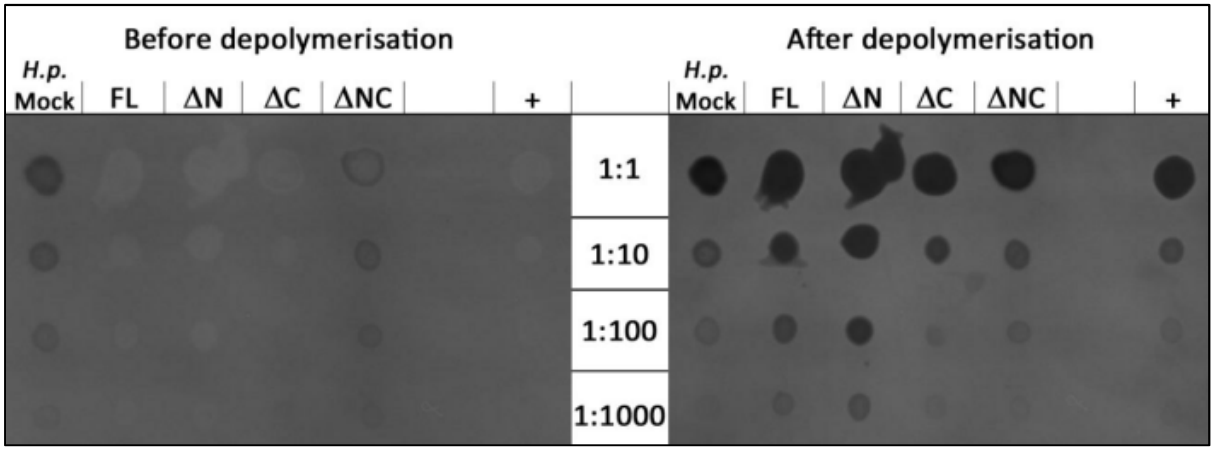

Fig. 6: CDA activity assay of PaCDA variants lacking no (FL), C-terminal (ΔC), N-terminal (ΔN) or both (ΔNC) chitin binding domains, 1 µl samples of culture supernatants including negative (Mock) and positive control (+) using 0.1% glycol chitin, stained with Calcoflour White, gels before and after depolymerisation of chitosan [Gilleßen, 2012].

4.3.4. Comparison of PaCDA and PaCDA-ΔN

Gilleßen carried out the experiments described in this section. By comparing the deletion variants and the full-length PaCDA, Gilleßens experiments were restricted to PaCDA-ΔN only, because the other two truncated enzymes could not be produced and purified from *H. polymorpha* cultures as reported above. Gilleßen tested the CDA activity at a range of pH values and found the same curve progression with a pH optimum at 9-10. In contrast, he found differences regarding the temperatures tested. The curve of PaCDA-ΔN was shifted towards lower temperatures with a broad plateau between 30°C and 50°C, in comparison to the optimum observed for the full-length PaCDA at 50-60°C. Like for the pH optimum, the maximal acetate release was at the same extent for the temperature-dependent

activities. PaCDA-ΔN showed about the same CDA activity than PaCDA, not only on colloidal chitin and glycol chitin, but also with chitosan, when the enzyme amounts were adjusted equimolar. Different buffer compositions tested, namely Teorell-Stenhagen with citric, phosphoric and boric acid; triethanolamine and ammonium carbonate buffer, had no significant influence on activity for both enzymes.

4.3.5. Enzymatic / mass-spectrometric fingerprinting

Chitosan DA 50% was used for a fingerprinting experiment to compare the modes of action of the full-length and truncated enzymes. The substrate was deacetylated to a DA about 16% for both samples, measured by acetate release, and then hydrolyzed by sequence-specific chitinosanase from *Alternaria alternata* like described in the previous chapter.

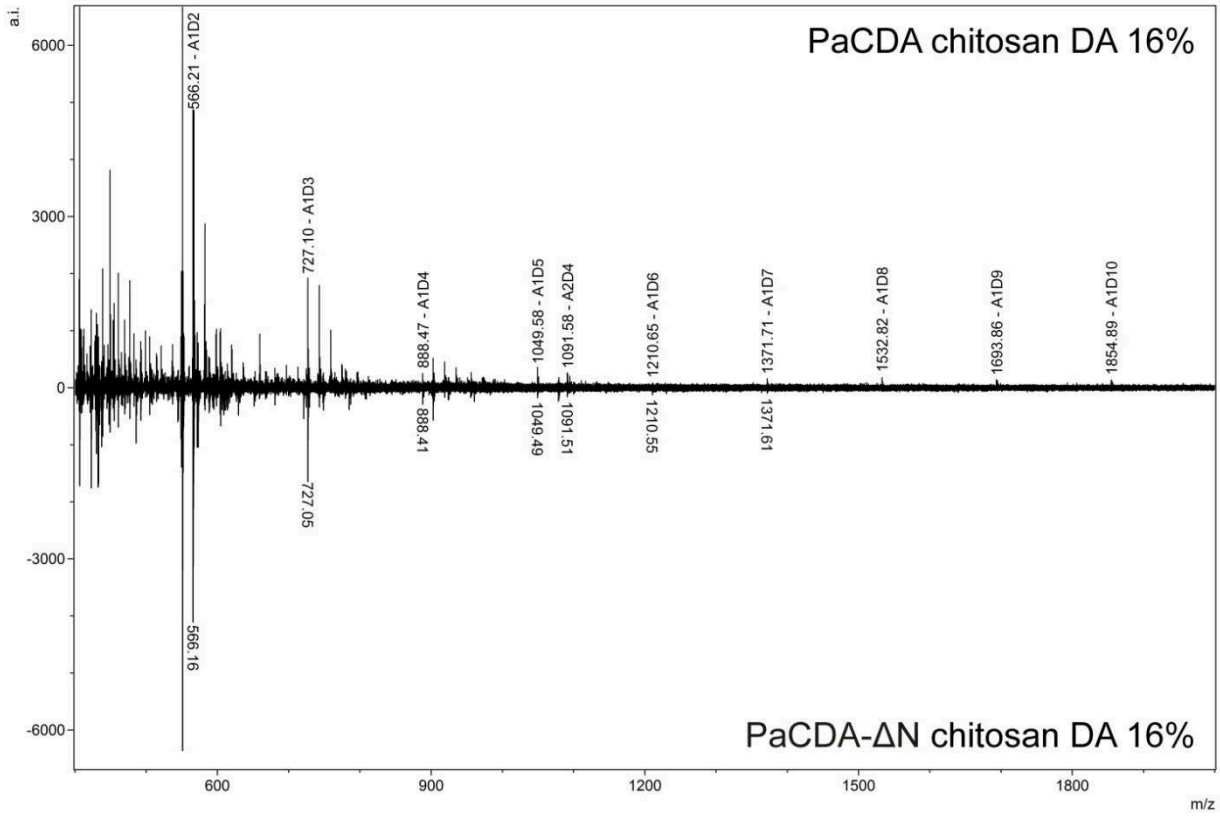

Fig. 7: Mass spectra fingerprinting, chitosan deacetylated by PaCDA (upper spectrum) or PaCDA N (lower spectrum) and subsequently hydrolysed with sequence-specific chitinosanase from *A. alternata*

By MALDI-TOF mass spectrometry analysis, the resulting chitosan oligomers could be identified and were annotated according to their composition of A and D units (Fig. 7). Except for A2D4, only

both spectra can clearly be seen in the additional peaks of larger oligomers (A1D8 to A1D10) for the full-length PaCDA, as the intensities are also similar. To summarize, we can say that both enzymes work in a common mode of action, but PaCDA produces larger D blocks.

4.3.6. PaCDA production in *E. coli*

After the purification of PaCDA-ΔC and PaCDA-ΔNC from *H. polymorpha* failed, a new strategy was followed with two improvements of for the truncated genes. First being the incorporation of the full C-terminal low complexity region to get a flexible linker and second the usage of the Twin-Strep-tag® for an enhanced Strep-Tactin binding (Fig 8). The Twin-Strep-tag was recently developed by IBA for a higher affinity, by combining two Strep-tag sequences with a linker of 12 amino acids.

First, the full-length PaCDA coding sequence was cloned into the pET-22b(+) vector for testing heterologous production in the *E. coli* T7-system using the resulting plasmid pET-22b(+)::PaCDA-StrepII. This plasmid was also used later for the introduction of the Twin-Strep-tag. When *E. coli* Rosetta 2 (DE3) [pLysS-RARE2] was used for the first production trial of full-length PaCDA, no Strep-tag signals were found by western blot in any fraction (data not shown). In the second trial, *E. coli* transformants carrying the additional plasmid pCDFDuet1::DSBC/TrxA were cultivated in up to 1 l auto-induction medium and tested for the production of the recombinant protein. In the first experiment, PaCDA could only be found in the insoluble fraction of the cell extracts as an overexpression band at about 35kDa in Coomassie-stained polyacrylamide gel and by western blotting. Then large-scale affinity purification was done by FPLC with Strep-Tactin matrix from the soluble fraction and thereby a small amount of PaCDA could be purified and detected on western blot, but only after the concentrated eluate was almost completely loaded onto the gel. To test the CDA activity, 1-µl samples were applied onto glycol chitin gels and positive signals could be seen for the crude extracts as well as the purified and concentrated samples.

This draws us to the conclusion that the production of PaCDA and their variants in *E. coli* is in principle possible, but limited by the fact that the major part is not folded correctly and ends up in inclusion bodies.

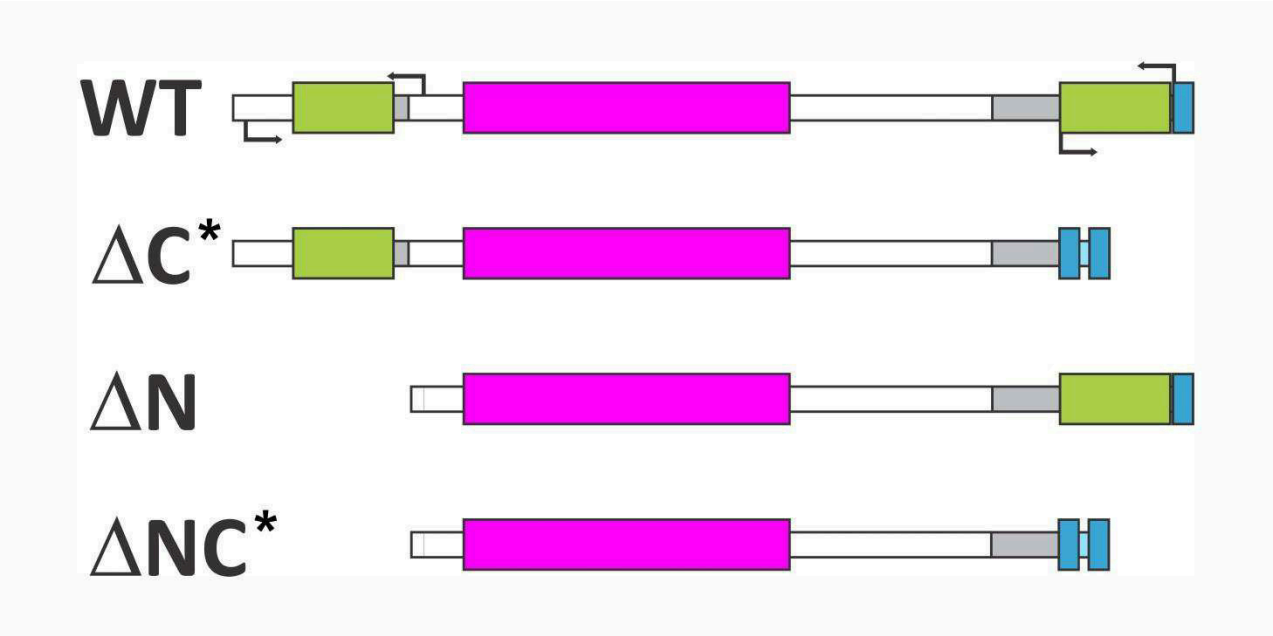

Fig. 8: Schematic structure of PaCDA and new truncated variants ΔC* and ΔNC* with lacking chitin binding domains (green), including the low complexity regions (grey) and Twin-Strep-tag (blue), polysaccharide deacetylase domain (pink), arrows indicate deletions.

4.4. Discussion

Chitin binding domains are modular structures that are known to help chitinases to bind their insoluble substrates, which are otherwise tough to access [Beintema, 1994; Iseli et al., 1993]. PaCDA described in this thesis is the first active CDA with two chitin binding domains and a good activity on colloidal chitin. As the two CBDs differ in sequence and length, it is conceivable that they also differ in their function. The aim of this study was to investigate the role of the CBDs in PaCDA by deletion of the C-terminal (ΔC), N-terminal (ΔN) or both CBDs (ΔNC) and the analysis of the truncated enzyme variants in comparison to the full-length PaCDA.

4.4.1. PaCDA-ΔN

The production and purification of PaCDA-ΔN was successful and yielded an amount of 2 mg protein per 1 l culture [Gilleßen, 2012] and also a high purity (Fig. 5A) equal to the full-length enzyme. The truncated enzyme showed the same activity level on glycol chitin and chitosan as well as on the insoluble substrate colloidal chitin. As the lack of only the N-terminal CBD does not result in a reduced activity, the C-terminal CBD is able to compensate the truncation or is enough to bind the insoluble substrate.

Therefore, colloidal chitin was used for testing the pH optimum, which was found to be at 9.0 for both enzymes [Gilleßen, 2012]. In my earlier studies I measured 10.5, but this result is in accordance to other extracellular CDAs like ClCDA, for which a pH optimum of 8, 8.5 and 11.5 was reported depending on the production strain [Shrestha et al., 2004; Tsigos and Bouriotis, 1995 and Tokuyasu et al., 1996; respectively]. The physiological optimum for all three enzymes is likely at 8.5, as discussed in the PaCDA manuscript (chapter 3.4.4).

In contrast, the temperature optimum measured for PaCDA (50-60°C) was the same as seen before (55°C), though there was a difference towards the deletion variant: PaCDA-ΔN showed also a broad activity curve, but an optimum of 40-50°C accompanied by more activity for the lower and less for the higher temperatures [Gilleßen, 2012]. This shift of the whole curve might be due to a structural artifact of the construct or a stabilizing effect of the CBD. If the deletion variant is also less stable at high temperatures, has to be tested in experiments carried out accordingly to the PaCDA assays.

Knowing that the general properties of both CDA versions were comparable, the next step was to test the mode of action. For this purpose, a high DA chitosan (50%) was used as substrate for an enzymatic/mass-spectrometric fingerprinting analysis, for that samples were hydrolyzed by the sequence-specific chitinosanase. By comparing the fingerprinting results (Fig. 7), differences were found in block length of several D units in a row. The two spectra could not be compared to solely-chemically produced chitosan with the same DP and DA, because a chitosan with a DA of 16% was not available at this time. This was also the lowest DA measured in this kind of experiment and is most probably due to over-incubation with a 4-fold higher amount of enzyme compared to the test described in the PaCDA chapter (3.2.5), but not confirmed by ^{1}H-NMR due to the low amount of sample. Nevertheless, these results are in accordance with the finding that PaCDA creates a non-random pattern with only single A units that cannot be further deacetylated. The discrepancy in product quality but not quantity is a first hint that the mode of action and PA produced could be different for both enzymes and therefore be influenced by the CBDs, leading to the assumption PaCDA might act on more multiple GlcNAc units on one chitosan chain than PaCDA-ΔN, but without being a real processive enzyme.

Additionally it has been found out that different buffer components, e.g. citric acid, did not have an inhibitory effect; however other properties like cofactors and inhibitors were not tested, yet. The

characterization in terms of substrate specificity and product analysis by ^{13}C-NMR spectroscopy like for PaCDA now have to be carried out for all truncation variants, ideally in parallel experiments. For PaCDA-ΔN it can be summarized, that in spite of the truncation a correct folding could be achieved and verified by activity tests, while PaCDA-ΔC was challenging to produce and the production of PaCDA-ΔNC failed.

4.4.2. PaCDA-ΔC and ΔNC

Like for the N-terminal truncation, the deletion variants lacking the C-terminal CBD were constructed using an alignment of PaCDA and ClCDA (Fig. 1), in a way that PaCDA-ΔC and ΔNC also ended with an alanine and the low complexity region was deleted along with the CBD. It was also taken into account that heterologously produced ClCDA had no additional amino acids as linker to the C-terminal His_6-tag, too [Shrestha et al. 2004]. In contrast to PaCDA-ΔN, the deletion of the C-terminal CBD caused problems during production, as it seemed to have a huge impact on correct folding and Strep-tag accessibility.

The initial screenings were done by western blot (Fig. 4) and the two C-terminal deletion variants yielded neither signals in the supernatant nor in the soluble, but in insoluble cell fraction samples. At this point it was clear, that PaCDA-ΔC and ΔNC were not properly produced as expected. When the CDA activity was assayed in the first screening with pools of the variants, CDA activity was found for all samples, and the control strain seemed to have only chitinase activity (Fig. 3). This suggested that at least a small amount of recombinant enzyme was present and the possibility to purify it from one of the fractions. After the failure of all purification trials the detailed analysis of protein production by Gilleßen showed that PaCDA-ΔC was produced in low amounts only and ΔNC was not produced at all (Fig. 5). Importantly the detection of CDA activity in the *H. polymorpha* control strain on the second activity gel (Fig. 6) urged the demand to revisit earlier results.

This activity has never been seen before, probably because it was masked by chitinase activity, and clearly demonstrated the limits of glycol chitin gel dot assays, besides the absolute necessity to include a negative control. In the case reported here it is conceivable that the white coloration of the gel before depolymerization caused by the CDA activity was overlaid by the chitinase activity resulting in dark spots [Trudel and Asselin, 1990]. The outcome of this is that it is hardly possible to detect slight

CDA activity, when there is also a chitinase present and the other way around. For this case a semi-native PAGE would be suitable to separate the different enzymes and a substrate containing overlay gel to specify their activity. The finding of CDA activity in *H. polymorpha* Mock (RB11 strain) was then confirmed by measuring acetate release after the incubation of culture supernatants with colloidal chitin.

The discrepancy of CDA activity found between the different experiments of this and also earlier studies raises the question if this CDA is only produced under certain, maybe stress-related conditions. For other yeasts it was reported that CDAs are involved in ascospore formation, which was shown for both CDAs of *Saccharomyces cervisiae* [Christodoulidou et al., 1996] and for one from *S. pombe* [Matsuo et al., 2005]. Assuming the production of heterologous proteins causes a high stress level, the expression pattern of these cultures could change towards stress adaption or spore formation. The genome of recently sequenced *H. polymorpha* strain DL-1 contains two putative CDA genes [Ravin et al., 2013], which were not investigated by now. By some means the cultivation of the *H. polymorpha* Mock strain by Gilleßen has induced the expression of this CDA for the first time [Gilleßen, 2012]. As this opens a new topic for investigation, it also raises the awareness for a proper purification of recombinant CDAs from *H. polymorpha*.

As the presence of PaCDA-ΔC was confirmed by activity tests and low amounts on acrylamide gel, it still remained unclear why it could not be purified or detected via the Strep-tag. The first and foremost explanation for this is that the Strep-tag is not accessible, due to a protein folding leading to steric blocking or incorporation to the catalytic domain. Certainly, the main portion of the single C-terminal deletion as well as the double deletion variant is folded or processed incorrectly and therefore ends up in the insoluble fraction (Fig. 4). It can be excluded that the lacking secretion is caused by a failure of the signal peptide, because the closer and first translated N-terminal deletion part did not show any secretion problems. The other purification trials most probably failed because of the low amount of PaCDA-ΔC produced and even more by the confusion with the *H. polymorpha* CDA in the culture supernatant.

4.4.3. PaCDA production in *E. coli*

Escherichia coli is not only an easy to handle and straight-forward expression system, but also has the advantage to have no own CDA activity. The *E. coli* chito-oligosaccharide deacetylase *chbG* differs

from fungal CDAs, as is active on chitobiose and chitotriose [Verma and Mahadevan, 2012]. *E. coli* was tested as an alternative expression system to *H. polymorpha* for the production of PaCDA and the deletion variants. New constructs of PaCDA-ΔC and ΔNC were designed based on the former results (Fig. 8), but were not investigated further, yet. The low complexity region of PaCDA is predicted to be a flexible linker between the catalytic domain and the C-terminal CBD and therefore could play this role also for affinity-tag binding. In contrast to *H. polymorpha*, *E. coli* is known to express biotin carboxyl carrier protein with affinity to Strep-Tactin which could be detected, so that the Twin-Strep can be an improvement for the affinity of the recombinant enzyme.

To test the ability of the prokaryote to produce the fungal enzyme, first the full-length PaCDA gene was brought into *E. coli* Rosetta 2 (DE3) cells, which carry the pLysS-RARE2 plasmid for rare codons. Recombinant protein as well as CDA activity could be seen, but only with the additional use of pCDFDuet1::DSBC/TrxA, not with pLysS-RARE2 alone, which implies that thioredoxin A and the disulfidebridge isomerase are needed for correct folding. By using these bimolecular tools, it was proven that the production is indeed possible, but the protein remains mainly insoluble and the amounts are too low for any further experiments. One could argue that the production might be better without the CBDs, because the correct folding of proteins containing lesser disulfide bonds is more likely. For ClCDA [Tokuyasu et al., 1999] and the diacetylchitobiose deacetylase from *Pyrococcus horikoshii* [Mine et al., 2012] it was possible to obtain refolded enzymes from inclusion bodies, but due to the CBDs I estimated the chances for PaCDA very low. In conclusion, *H. polymorpha* has proven to be a suitable expression system for PaCDA and therefore should also be for the newly constructed truncation variants.

4.4.4. Conclusion and Outlook

The generation of the deletion constructs followed the same strategy as used earlier for the chitinases; however, production of two of the three enzyme variants in *H. polymorpha* was more challenging than expected. The characterization of PaCDA-ΔN could be initiated and the results obtained are promising. This is due to similar properties to the full-length enzyme with only slight differences in thermal stability and, unexpectedly, due to indications of a different mode of action. Although PaCDA-ΔC and ΔNC could not be produced and purified, the results led to the designing of new constructs PaCDA-ΔC* and ΔNC* differing at the C-terminus (Fig. 8), which still have to be investigated. After the finding that *E. coli* is able to produce full-length PaCDA, but in quantities significantly lower as

obtained from *H. polymorpha*, the latter host still represents the expression system of choice. The discovery of an active CDA naturally expressed in *H. polymorpha* has to be taken into account for further experiments, but could also be a target for characterization studies.

After the obstacles of protein production have been overcome, further studies on the characterization of the three CBD deletions could clarify the role of the CBDs for PaCDA. The production of all truncation variants using the same expression system will be beneficial for the comparability. When CDA activity of the CBD deletions is confirmed, binding assays and comparative activity tests using all possible substrates available are the first experimental steps to be addressed. If PaCDA-ΔC and ΔNC have similar activity properties like PaCDA and PaCDA-ΔN, fingerprinting experiments and ^{13}C-NMR spectroscopy will provide an insight into the mode of action with and without each CBD. To investigate the hypothesis that the CBDs have different binding preferences, the modules could be solely produced fused to a marker protein like the Green Fluorescing Protein. The fluorescing tag can then be used for quantifying the binding of the fusion protein towards different substrates. Further experiments should also address the direct comparison of PaCDA-ΔNC with ClCDA, which is the most similar known CDA, so that also a domain swap is imaginable.

PaCDA is not only the first active CDA with two CBDs, but generates a non-random pattern of acetylation on chitosan as well. Hence, the role of the CBDs in this process is a topic that requires further investigation.

5. Discussion

The preparation of chitosan from chitin by chitin deacetylases (CDAs) versus chemical deacetylation has already been discussed in 2000 by Tsigos et al.. The advantages of a controlled enzymatic reaction still are opposed to the low efficiency towards crystalline chitin of all known CDAs. Zhao et al. [2010] discussed that the deacetylation could only be improved by substrate modifications and that the substrates typically used by researchers for their enzymatic CDA reactions originate from various sources and are often poorly characterized. Weinhold et al. [2009a] analyzed commercially available chitosans in a multi-dimensional matter and reported differences in molecular weight (DP), polydispersity, hydrodynamic radius, intrinsic viscosity and degree of acetylation. He and his colleagues showed that also the PA value for a large set of chemically produced chitosans varies, but all reveal a random-dominated pattern of acetylation [Weinhold et al., 2009b].

The application of those commercially available chitosans in biological systems like plant protection and medicine led to contradictory and sometimes questionable results [Aam et al., 2010; Hadwiger, 2013]. But if we consider that chitosans occurring in nature are typically generated from chitin by CDAs, the role of PA for biological activities becomes considerable. Like other enzymes, CDAs possess a discrete catalytic center surrounded by a certain subsite structure for substrate binding and act in a specific mode of action that enables the generation of non-random PAs. CDAs creating a specific PA on oligomers and their processivity have been demonstrated earlier [Tsigos et al., 2000].

In the present study, a new type of CDAs was investigated for the production of chitosans with non-random PAs different from known chitosans, possessing additional chitin binding domains (CBDs) in a multi-domain structure. Within the increasing number of CDAs being characterized so far, no CDAs containing CBDs have demonstrated activity yet. At the same time, enzymes deacetylating crystalline chitin like it can be achieved by chemistry are not described until now. Because CBDs are commonly found in chitinases degrading crystalline chitin [Beintema, 1994; Iseli et al., 1993], CDAs with CBDs might be more effective on this substrate than those containing none. In the present study, the identification, heterologous protein production and characterization of novel CDAs including CBDs was investigated. The role of the CBDs for enzyme activity, substrate preferences and mode of action was addressed by deletion variants lacking CBDs and the chitosan products were analyzed for PA and biological activities.

5.1. Heterologous production of chitin deacetylases with chitin binding domains

In this study, chitin binding domains present in a CDA gene are a very special feature and at the same time represent the major challenge for heterologous protein production. These CBDs contain four highly conserved disulfide bridges each that need a reducing environment for bonding. Using yeast expression systems like *Schizosaccharomyces pombe* and *Hansenula polymorpha*, owning a eukaryotic secretory pathway, seem a reasonable choice for fungal CDAs, but in the case of *S. pombe,* recombinant protein production was not successful. On the other hand *Escherichia coli* is a widely applied expression host and different strategies for the production of proteins with disulfide bonds are available [de Marco, 2009]. The application of the three different species mentioned above for heterologous expression of CDAs containing CBDs will be discussed in the following chapters.

5.1.1. *Escherichia coli*

E. coli has successfully been used for production of recombinant CDAs from *Colletotrichum lindemuthianum* (ClCDA) [Tokuyasu et al., 1999], *Aspergillus nidulans* [Wang et al., 2009] and the archeon *Pyrococcus horikoshii* [Mine et al., 2012]. For the protein production by *E. coli* in this study, the strain Rosetta 2 (DE3) carrying the pLysS-RARE2 plasmid for stress-induced autolysis and seven codons rare in *E. coli* [Novy et al., 2001] was chosen. The autoinduction medium described by Studier [2005] was used for all production cultures for a smooth induction and higher yields. The strategy followed was focused on cytoplasmic protein production including biotechnological modifications, not on secretion into the naturally oxidizing periplasm. One modification is based on thioredoxin 1 (trxA) that reduces cysteines of disulfide bonds in the cytoplasmic redox system. This mechanism functions only when trxA is constantly reduced by thioredoxin reductase, an overexpression of trxA results in performing the opposite task on co-expressed proteins [de Marco, 2009]. Therefore, BC95 was constructed as a fusion protein with an N-teminal thioredoxin [Lavallie et al., 1993] and PaCDA was co-expressed with trxA [Yasukawa et al., 1995]. Active PaCDA could be purified from *E. coli* in a small amount, but BC95 was not produced at all and not even detected in insoluble cell fractions. The reason for successful protein folding resulting in active enzyme might be size or structure of the PaCDA compared to BC95, but also likely the additional co-expression of DsbC. The disulfide bond isomerase DsbC is responsible for oxidative protein folding in the periplasm [Messens and Collet, 2006], while the cytoplasmic overexpression can help for a higher yield of soluble protein [Berkmen, 2012]. In summary, the “foldase” DsbC and the reducing environment provided by trxA were required

for folding PaCDA, but the majority of the protein remained insoluble and the amount of active PaCDA purified was too low to quantify, concluding *E. coli* was not suitable for fungal CDAs with CBDs at least in these two cases (s. chapters 2 and 4).

All CDAs mentioned above for successful production in *E. coli* were renatured from inclusion bodies *in vitro*, including ClCDA showing two disulfide bonds in the crystal structure [Blair et al., 2006]. In the *A. nidulans* CDA protein sequence one cysteine pair is absent in the catalytic domain, but present in PaCDA and BC95. Altogether, PaCDA with two CBDs possesses 10 disulfide bonds and BC95 containing three CBDs carries even 14. Statistically, the more S=S bridges a protein contains, the more unlikely it becomes to fold spontaneously correct *in vitro*, thus this is no option for CDAs containing one or more CBDs. Even with the use of CsbC *in vivo* it could be shown that not only the number but also the pattern of disulfide bond has an influence on correct folding [Berkmen, 2012].

A further strategy for correct folding of disulfide-bond-rich proteins is the combination of thioredoxin reductase (*trxB*$^-$) and glutathione reductase (*gor* $^-$) mutations in the strain FÅ113 (*trxB, gor, ahpC**), commercially available as Origami^{TM} by Novagen. It was successfully used [Bessette et al., 1999], but grows slow due to the oxidizing cytoplasm and is not able to refold mis-oxidized proteins being the task of periplasmic CsbC [Ito and Inaba, 2008]. Therefore, the Shuffle® strain was developed by New England Biolabs able to correctly fold disulfide bonded proteins by an additional CsbC in its cytoplasm [Lobstein et al., 2012], and was recently applied successfully to a thioredoxin fusion protein [Kong and Guo, 2014].

Although for improvement of protein solubility the use of fusion to maltose binding protein was adopted [Tait and Straus, 2011], another disadvantage of prokaryotic protein production is the absence of glycosylation that can lead to insoluble protein. In non-zygomycetic fungi CDAs are extracellular enzymes and often glycoproteins [Zhao et al., 2010].

5.1.2. *Schizosaccharomyces pombe*

The fission yeast *S. pombe* was the first expression system used for the heterologous production of BC95, because six possible N-glycosylation sites were predicted in the BC95 protein sequence. The glycosylation of the BC95 protein might be required for the production of soluble and active enzyme and can be provided by the unicellular eukaryote. In contrast to the widely applied host *Saccharomyces cerevisiae,* the fission yeast is conducting less hyperglycolsylation, which is an advantage because it might slow down secretion and cause low yields [Celik and Calık, 2012]. *S.*

pombe strain *ura4*$^-$ D18 and the vector pMel have successfully been used (in our lab) for other eukaryotic enzymes, namely a CDA from *Puccinia graminis* sp. f. *tritici* (PgtCDA) [unpublished results] and the human chitotriosidase [Kalagara, 2013]. Nevertheless, this system has the disadvantage of requiring cultivation in minimal medium containing no uracil to retain the plasmid containing an *ura4*$^+$ marker for the auxotrophic *ura4*$^-$ D18 strain [Grimm et al., 1988]. For protein production the cells have to be transferred into medium without thiamin for inducing the *nmt1* promoter [Maundrell, 1993]. Later, the trial to isolate the expression plasmid from the yeast cells was not possible; indicating that the successfully expressing PgtCDA clone had integrated the plasmid into its genome. If the strategy of stable integration into the host genome is applied on purpose, it might overcome the drawbacks mentioned above.

A current development in biotechnology also applied on *S. pombe* is the generation of minimum genome factories, because the fission yeast has one of the smallest genomes amongst eukaryotes [Giga-Hama et al., 2007; Kumagai et al., 2014]. In doing so, all genes not required for growth in (low cost) complex media are deleted and strains are analyzed for growth and metabolism to create expression hosts with high production efficiency [Sasaki et al., 2013]. For this objective also the deletion of proteases and the improvement of protein secretion were investigated [Idiris et al., 2010].

5.1.3. *Hansenula polymorpha*

H. polymorpha is a methylotrophic yeast that is used for extracellular production of recombinant proteins in industrial-scale, because the low amount of yeast own proteins secreted into the culture medium is beneficial for downstream processes [Stöckmann et al., 2009]. In comparison to the *S. pombe* system described above, genes heterologously expressed by *H. polymorpha* are mitotically stable integrated into the genome. This process called passaging and stabilization is time-consuming but allows a reproducible cultivation under non-selective conditions using inexpensive full media [Gellissen, 2000]. An advantage over the widely used methylotrophic *Pichia pastoris* is that *H. polymorpha* does not require

toxic and flammable methanol for inducing protein production, because the *FMD* (formate dehydrogenase) promoter used is induced by methanol and repressed by glucose, but de-repressed by glycerol [Hollenberg and Gellissen, 1997].

The heterologous expression of BC95 yielded soluble protein, whereas it was only detected intracellular indicating difficulties in the secretion mechanism. The secretion signal used in this study

was derived and engineered from *S. cerevisiae* MF-α1 (mating factor α) and is a widely used leader sequence also for *H. polymorpha* [Kurjan and Herskowitz, 1982]. This pre-pro leader peptide initiates the two-step maturation of the recombinant protein by translocation into the ER for folding and glycosylation. As BC95 protein was found soluble and glycosylated, the bottleneck of production seemed to be the second step of exporting the pro-protein into the Golgi for glycan maturation and subsequent secretion into the medium [Delic et al., 2013]. In comparison to PaCDA containing no N-glycosylation sites, six possible N-glycosylation sites were predicted in the BC95 protein sequence that could be a source of errors.

The production of PaCDA and PaCDA-ΔN in *H. polymorpha* was successful and pure enzymes were obtained by Strep-Tactin affinity chromatography; results regarding the enzymes themselves are discussed in chapter 5.2.2. The deletion variants PaCDA-ΔC and ΔNC share the C-terminal CBD deletion, which is most probably responsible for the failure in production of the two enzymes. Although the sequences of the C-terminus from ClCDA and PaCDA-ΔC/ΔNC were similar (Fig. 1, chapter 4.3.1), the C-terminal truncation of PaCDA enzyme led to a low yield of active PaCDA-ΔC and no protein detectable for the double deletion. A strategy to overcome the misfolding of these two constructs might be to retain the linker region at the C-terminus, which also ensures the accessibility of the Strep-tag (see chapter 4.4.2).

The StrepII-tag was applied as C-terminal fusion to all recombinant proteins in this study, with the exception of the first BC95 experiment that was carried out using a His_6-tag. Recombinant CDAs described in literature were produced by using His-tag fusions [Shrestha et al., 2004; Wang et al., 2009; Yamada et al., 2008], but it cannot be excluded that the His-tag might interfere with the zinc ion of the CDA active site, as seen for the ClCDA crystal structure [Blair et al., 2006]. Therefore, we constructed the recombinant enzymes including StrepII protein sequence, which usually not interferes with folding [Schmidt and Skerra, 2007].

Furthermore, it has to be stated that *H. polymorpha* possesses CDA activity discovered in this study, but this was no obstacle for the production of PaCDA and PaCDA-ΔN. Like other yeasts, *H. polymorpha* owns two CDA genes [Ravin et al., 2013], which were not analyzed so far. As discussed in chapter 4.4.2, the CDA activity appeared in only some of the experiments and the reason for enzyme expression remained unclear.

Having investigated three different systems for heterologous protein production, it can be said in summary that the *S. pombe* system used was not suitable at least for BC95. Whereas it was possible

to produce PaCDA in an *E. coli* system with modifications for disulfide bond formation in the cytoplasm and this might be further enhanced by using the newest tools available. But in comparison to the production efficiency of the yeast *H. polymorpha* yielding more than 1 mg protein per 1 l culture, the *E. coli* system is not rewarding for the expression of CDAs with CBDs. Therefore, protein production in *H. polymorpha* should be continued using new constructs of PaCDA deletion variants and might be optimized for BC95 to analyze the enzymes function.

5.2. Characteristics of chitin deacetylases with chitin binding domains

Fungal CDAs have been divided into two groups based on their origin and natural function: The role of CDAs in zygomycetes was assigned to the assembly of the chitosan-rich cell walls by acting processively in tandem with a chitin synthase [Davis and Bartnicki-Garcia, 1984]. CDAs of asco- and basidiomycetes modify the fungal cell wall chitin after hyphal growth, either in plant-pathogen-interactions, the spore formation of yeasts or in another developmental stage [Tsigos et al., 2000]. CDAs from all three taxonomic groups have been characterized [Zhao et al., 2010] but until now no CDAs containing CBDs type CMB18, which are limited to ascomycetes. In the present study two CDAs with CBDs were heterologously produced, one from *Botrytis cinerea* and one from *Podospora anserina*, their role and functions will be presented in the following.

5.2.1. Botrytis cinerea putative CDAs

B. cinerea is a plant pathogen, thus the function of one of the six putative CDAs expressed during conidia germination shown in this study might be viewed as infection strategy like reported for other plant pathogenic fungi [El Gueddari et al., 2002]. However, the chitin content of *B. cinerea* mycelium three to nine days after inoculation in axenic culture was estimated to be only between 1% and 5% [Cantu et al., 2009]. Though the chitin content of spores might be higher, because the thick conidia cell wall consists of two layers, of which the outer one is broken during germination [Hawker and Hendy, 1963].

As reported in the *B. cinerea* manuscript (chapter 2.3.2), the BC95 gene was upregulated 4 h post inoculation (hpi) in the presence of 10 mM fructose. Recently, Leroch and co-workers have investigated transcriptome profiling of germinating *B. cinerea* conidia and revealed BC95, referred as *cda1*, as reporter gene for germination due to its strong upregulation before germ tube appearance at 1 hpi [Leroch et al., 2013]. They found germination to be more rapid on apple wax-coated surfaces,

starting appressorium development at 4 hpi in fructose containing medium, than on glass used in the present study. Nevertheless, analysis of the *cda1* promoter by a GFP reporter strain demonstrated that expression was independent of the germination conditions and correlated with the outgrowth of germ tubes. In the same publication the occurrence of BC1G_03291 (*cda2*) and BC1G_06509 (*cda3*) was also mentioned [Leroch et al., 2013].

For biotrophic pathogens like *Colletotrichum graminicola* and *P. graminis* it is important for successful infections to prevent a hypersensitive reaction of the host plant triggered by elicitors like chitin oligomers [Kombrink et al., 2011]. Belonging to the different taxonomic groups of asco- and basidiomycetes, both species have convergently evolved a pathogenicity related mechanism, which protects the cell wall chitin from degradation by plant chitinases. They possess CDA activity that changes the surface of infection hypha from chitin into chitosan directly after the penetration pegs develop from appressoria [El Gueddari et al., 2002] and is also able to deacetylate chitin oligomers [unpublished results]. Because *B. cinerea* is a necrotrophic pathogen, its infection mechanism differs [Choquer et al., 2007] as it does not have to hide from the plant immune system, but rather makes use of reactive oxygen species and induces programmed cell death [Govrin and Levine, 2000; Williamson and Tudzynski, 2007]. These facts considered together with the expression of BC95 during early germination bring us to the assumption that this enzyme is more likely involved in changing the spore wall structure for hyphal outgrowth than in plant infection.

The activity of BC95 was analyzed using samples of germinating *B. cinerea* conidia at different time points and after heterologous protein production described above. However, CDA activity was not detected in *B. cinerea* samples assayed using glycol chitin containing gels, although it might be present in the mycelium fraction that also showed chitinase activity (chaper 2.3.3, fig. 3). The disadvantages of this activity assay concerting multiple activities have been discussed in 4.4.2. By using proteomic tools, Espino and colleagues have investigated the proteins secreted during the first 16 h after germination of *B. cinerea* in liquid culture, wherein germ tubes but no appressoria developed [Espino et al., 2010]. The secretome contained different polysaccharide hydrolases and other degrading enzymes, but no CDA. But taking into account the method using a dialysis bag for separating the conidia from the surrounding medium, it is supposable that a CDA tightly attached to the fungal cell wall by chitin binding domains might not be detectable in the supernatant.

The heterologous expression of BC85 in *H. polymorpha* yielded soluble protein, but as it was found intracellular, it might not represent the enzymatically active form. The activity of recombinant BC95 was tested using chitosan DA 56%, colloidal chitin and chitin pentamer as substrates for acetate release measurements and using glycol chitin in a gel assay, but was not proven. Still, the occurrence of a polysaccharide deacetylase domain and chitin binding domains within the same protein sequence does not necessarily signify that the resulting enzyme is a CDA. To clarify this, other polysaccharide substrates should also be analyzed in further experiments, e.g. xylan and peptidoglycan based on the catalytic domain [Caufrier et al., 2003] or fungal cell walls, as chitin was shown to be covalently linked to the glucans [Kollar et al., 1995].

While the particular function of BC95 remains unclear, it is certainly connected to conidia germination. Interestingly, a homolog is present in the closely related fungus *Sclerotinia sclerotiorum*, which lacks the production of macroconidia along with the whole asexual cycle [Amselem et al., 2011]. If the function of BC95 is also related to the germination of ascospores, it might be demonstrated by an *in situ* chitosan staining using chitosan affinity protein [Nampally et al., 2012].

5.2.2. Podospora anserina CDA

P. anserina is a coprophilous ascomycete lacking the asexual cycle and being heterothallic, hence, model fungus for aging/senescence and mating type [Osiewacz, 2002]. As a late grower on herbivore dung, it possesses a different set of carbohydrate degrading enzymes like cellulases, compared to the pectin degrading *Aspergillus* species growing earlier [Coutinho et al., 2009]. The large number of GH18 and CBM18 domains, 20 and 30 respectively, in the genome of *P. anserina* [Espagne et al., 2008] and the discovery of a new broad-specificity β-glycanase containing one usually cellulose binding CBM1 might indicate that chitin and glycan degradation might also be important for *P. anserina.* The source of chitin and glycan would be cell walls of other fungi [Bartnicki-Garcia, 1968] growing earlier on the dung [Lafond et al., 2012]. The *P. anserina* genome also contains six sequences predicted to be xylanases or CDAs due to their polysaccharide deacetylase domains. In a recent study, one CDA (GenBank: CAP60162) was detected by secretome analysis following a cultivation with Avicel, a microcrystalline cellulose powder, but not with sugar beet pulp, rich in pectins [Poidevin et al., 2014]. This secreted CDA is identical to PaCDA reported in this study, but studies on expression patterns of *P. anserina* CDA genes have not been carried out. Concerning the role of

CDAs for *P. anserina,* it has to be considered that this species is not a zygomycete, neither a yeast and non-pathogenic, but belongs to the Sordariomycetes, which also contains plant pathogens. It was reported that its tetraspanin-like protein *PaPls1* is required for germination of heavily melanized ascospores and has a functional ortholog (*MgPLS1*) in *Magnaporthe grisea* [Lambou et al., 2008]. *MgPLS1* is necessary for pathogenicity of *M. grisea* by acting for the differentiation of penetration pegs from melanized appressoria [Clergeot et al., 2001], a structure not present in *P. anserina.* Assuming the same molecular mechanism is underlying the two processes, this might also apply to a CDA acting on the own cell walls, which is important for some plant-pathogenic fungi [El Gueddari et al., 2002]. In conclusion, the secreted CDA might play a role in hypothetical chitin degradation or is acting on the *P. anserina* own cell wall.

5.2.2.1. PaCDA characteristics

The recombinant PaCDA presented in this study was successfully produced in *H. polymorpha* and had clear similarities to the well described CDA from the plant pathogen *C. lindemuthianum*. The PaCDA gene was identified by an alignment of putative CDA protein sequences and the sequences of biochemically characterized CDAs, revealing that PaCDA was most similar to ClCDA in amino acid sequence. The biochemical characterization of PaCDA yielded an optimal temperature of 55°C and an optimal pH of 8.5. These values are not only similar to the characteristics of ClCDA [Shrestha et al., 2004] but also to those of other CDAs belonging to the extracellular type [Zhao et al., 2010]. Like other enzymes in this group, PaCDA is not inhibited by acetate, though acetate inhibition was reported for the intracellular CDAs of zygomycetes [Gao et al., 1995; Kafetzopoulos et al., 1993]. For ClCDA, it was demonstrated that Zn^{2+} was a tightly bound cofactor in the catalytic site of the enzyme [Blair et al., 2006]. I have seen consistent results for the zinc cation in PaCDA, which was enclosed in a way being not accessible for EDTA, because PaCDA was not inhibited by the chelator and the presence of other bivalent cations had only minor effects.

For the analysis of substrate specificity, different chitinous substrates have been assayed for deacetylation measurement via acetate release detection. The minimum substrate for PaCDA is the chitin dimer (DP 2), but deacetylation was found to be slow on chitin oligomers. It is supposable that the CBDs do not take part in the deacetylation of chitin oligomers but in the enzyme activity towards insoluble crystalline substrates. So far, the ability of CDAs to deacetylate different insoluble chitin substrates was not depending on the enzymes themselves, but rather on the substrate crystallinity that

was changed by various chemical treatments in the studies [Beaney et al., 2007; Cai et al., 2006; Pacheco et al., 2013]. PaCDA possessed activity towards α-, β- and colloidal chitin, however, the change in DA was comparable to those reported for other CDAs, indicating that the deacetylation was limited to the substrate surface as well. While PaCDA not yielded the desired result of complete deacetylated products, the obtained chitin particles might still represent an interesting material for applications e.g. as column matrix material [Aye et al., 2006]. The analysis of deletion variants concerning the role of CBDs for PaCDA and the deacetylation of crystalline chitin will be discussed in chapter 5.2.2.3.

The deacetylation of different chitosans with DAs ranging from 9% to 61% by PaCDA resulted in different DAs afterwards (chapter 3.3.3, fig. 3A) suggesting that a specific PA is produced, which is a poorer substrate for the enzyme than chemically produced chitosan of the same DA. This result was the starting point of my research on the mode of action of PaCDA that was carried out using a chitosan polymer.

5.2.2.2. PaCDA mode of action

The mode of action on chitin oligomers has been demonstrated for ClCDA [Tokuyasu et al., 2000a] and the CDA from *Mucor rouxii* (MrCDA) [Tsigos et al., 1999], but using polymers, the processive mode was only proven for MrCDA [Martinou et al., 1998]. In the present PaCDA study, initial mass-spectrometric analysis of oligomers indicated full deacetylation in a presumably random fashion with subsite preferences similar to the results for ClCDA [Hekmat et al., 2003], whereas further analysis for revealing the mode of action was focused on polymers.

For this purpose, two chitosans were generated using the same starting material of DA 0% by reacetylating to DAs of 33% and 61%. The subsequent deacetylation of chitosan DA 61% by PaCDA to DA 29% resulted in two chitosans with the same DP and DA but potentially different PAs. The comparison of these two chitosans by enzymatic/mass spectrometric fingerprinting analysis with highly sequence specific *A. alternata* chitinosanase [Beneteau et al., in preparation] yielded indeed differences in composition and abundance of the obtained oligomers. The spectrum of chemically produced chitosan contained more peaks and larger oligomers, whereas the spectrum of PaCDA deacetylated chitosan showed higher intensities for oligomers with only one GlcNAc unit, for example A1D2 and A1D3 (chapter 3.3.5, fig. 5). The occurrence of less GlcNAc units next to each other in

enzymatically produced chitosan indicates that PaCDA requires two side-by-side GlcNAc units for deacetylation and hints to a PA more regular than random.

After this finding, both chitosans were analyzed by ^{13}C-NMR to investigate the distribution of GlcNAc units along the chitosan polymer represented by the experimental diads AA, AD, DA and DD of C5 carbon atom peaks [Varum et al., 1990; Vårum et al., 1991]. The result of this measurement was in agreement to the fingerprinting results by showing a smaller AA peak for the PaCDA chitosan (chapter 3.3.5, fig. 6). By applying the formula established by Weinhold et al. to the obtained diad frequencies, the P_A of PaCDA-treated chitosan resulted in 1.33, being the highest value determined so far by this method [Kumirska et al., 2009; Weinhold et al., 2009b]. The value for chemically-reacetylated chitosan was 1.11 and thereby in the range of P_A values found for chemically produced chitosans with random-dominated PAs. Whereas the processive mode of MrCDA is yielding a more blockwise PA [Martinou et al., 1998], the PaCDA-generated PA presented in this study is to my best knowledge the first proven alternating pattern on chitosan polymers. As mentioned above, the reason for such PA is most probably due to the subsite structure demonstrated for ClCDA with varying substrate preferences of each subsite [Hekmat et al., 2003]. The options for generating chitosans with non-random PAs and their properties will be discussed in chapter 5.3. In contrast to ClCDA, it is conceivable for PaCDA that the CBDs also play a role in mode of action and creating non-random PA.

5.2.2.3. Role of CBDs for PaCDA

Because PaCDA was the first CDA containing CBDs that was biochemically characterized, the role of the binding domains were investigated by comparing deletion-variants lacking the N-terminal (ΔN), the C-terminal (ΔC) and both domains (ΔNC) to the full length enzyme. As discussed previously, the production and purification of PaCDA-ΔC and PaCDA-ΔNC failed; for this reasons only the N-terminal deletion variant was used for comparative characterization and will be discussed in the following. PaCDA and PaCDA-ΔN deacetylated colloidal chitin to the same extent, but as discussed above, the activity of both enzymes was limited to the surface. CBDs of chitinases enhance the activity on crystalline chitin substrates [Iseli et al., 1993]; however, this effect was not detected for PaCDA since only the single deletion was tested and one CDA might be sufficient for binding. The crystal structure of a rice chitinase containing also one CBD of CMB family 18 was solved. The docking simulation with possible substrates suggested that this type of CBD is unsuitable for binding to crystalline α-chitin [Kezuka et al., 2010]. This can be explained by the pocket-like structure of type C CBMs, a group of

which some CBMs, including CMB 18, were originally identified as lectins [Boraston et al., 2004]. For PaCDA, we can conclude that the CBDs support the enzyme in substrate binding of amorphous chitin chains emerging into the surrounding solution, but cannot disrupt chitin crystals [Aye et al., 2006]. Respective to the physiological role of CBDs for *P. anserina*, they might help the secreted enzyme to stay close to the substrate it is naturally acting on.

Enzymatic/mass spectrometric fingerprinting analysis comparing PaCDA and PaCDA-ΔN revealed slight differences of the two spectra (chapter 4.3.5, fig. 7) indicating that the chitin binding domains might also play a role for the mode of action. The discrepancy was found in the size of the oligomers obtained, such as PaCDA produced larger oligomers with more deacetylated units in a row. This was leading to the assumption that PaCDA stays close to one chitosan chain and might even deacetylate multiple GlcNAc units in a row supported by the CBDs, while unproductive bindings would explain the single GlcNAc units left. A mode of action, forming also unproductive bindings depending on the subsites was demonstrated for the processive exo-chitinase B (ChiB) from *Serratia marcescens*, which is moving the sugar chain two residues at a time [Sørbotten et al., 2005]. This is explicable by picturing the three-dimensional structure of a β-1,4-linked chitin chain, who's N-acetylgroups of GlcNac units next to each other are pointing in opposite directions. Another Hypothesis based on the differences of the two CBDs indicated that one CBD might possess affinity to chitosan, which would assist the enzyme moving along the polymer chain. Mine and co-workers solved the crystal structure of ChBD1, a CBM family 5, from a *Pyrococcus furiosus* chitinase and revealed a different structure and binding affinity in comparison to ChBD2 from the same enzyme [Mine et al., 2014; Nakamura et al., 2008]. Hence, they suggested that the two CBDs each play a different role in chitin binding. A similar assumption was made for an *Aeromonas hydrophila* chitinase containing different N- and C-terminal CBDs [Wu et al., 2001].

However, the products of PaCDA obtained during the fingerprinting analysis using chitosan that was not deacetylated to the minimum (DA 30%) contained oligomers with up to four GlcNAc units (chapter 3.3.5, fig. 5). Therefore, and including the similarities to ClCDA, a processive mode of PaCDA is unlikely. Still, the presence of CBDs might influence the substrate binding additional to the subsites of the catalytic center. This has to be investigated in further studies that also analyze the variants PaCDA-ΔC and PaCDA-ΔNC and incorporates binding assays to chitin and chitosan.

Drawing a conclusion towards CDAs containing CBD presented in this study; the CDA activity of BC95 from the plant pathogen *B. cinerea* was not proven, whereas PaCDA from *P. anserina* is certainly a CDA. PaCDA showed similarities to characterized CDAs and was capable of producing a non-random PA on chitosan that has not been reported until now.

5.3. Chitosans with non-random pattern of acetylation

Chitosans possessing non-random PAs have been discussed for some time, but were rarely investigated so far [Tsigos et al., 2000; Zhao et al., 2010]. Only one study reported the analysis of an enzymatically-created chitosan by ^{13}C-NMR to prove the processive mode of MrCDA [Martinou et al., 1998]. This might partly be due to the large amount of sample needed for this measurement, in biological terms about 50 mg of substrate have to be deacetylated [Weinhold et al., 2009b]. In the present study, the generation of chitosans with non-random PA was demonstrated by the deacetylation reaction of PaCDA leading to an alternate-dominated pattern containing less GlcNAc units in a row than a random distribution. Assuming PaCDA owns the same subsite structure than ClCDA, the substrate preferences still could differ, hence, ClCDA might produce a different (non-random) PA. This implies CDAs are theoretically capable of producing random PAs as well as non-random PAs, even if they not act processively. This hypothesis was recently addressed by a master's thesis co-supervised by me, comparing the PAs on chitosan polymers from three different CDAs to chemically generated PAs [Wattjes, 2014]. In addition to the PA found for PaCDA described above, a CDA from *Pestaloziopsis spec*. created a random-dominated PA and a CDA from *P. graminis* f. sp. *tritici* resulted in a more blockwise pattern. Initial studies by Wattjes concerning physico-chemical properties, e.g. intrinsic viscosity and anti-microbial activity indicated different behaviors of the chitosans varying in PA, suggesting the charge distribution within the polymer is an important factor [Wattjes, 2014]. The PAs of ClCDA on chitosan polymers and of chitosans deacetylated by other zygomycete CDAs than from *M. rouxii* remain to be investigated. It is of importance to mention that the PA of different CDAs should be compared directly using the same substrate and a medium DA (e.g. 30%), due to the dependence of P_A versus DA [Weinhold et al., 2009b]. This study reported a tendency of chitosans with lower DAs to lower P_A values, which is most probably due to the NMR method itself, because the smaller the AA peak for low DAs becomes, the more imprecise is the calculation of its area compared to the surrounding peaks.

Another possibility for the production of non-random PA chitosan is the isolation from zygomycete mycelia, which was reported for *M. rouxii* [Trutnau et al., 2009] and *Absidia coerulea* [Wang et al., 2008b]. Although ^{13}C-NMR was used to identify the obtained polysaccharide as chitosan, the analysis of the PA is missing in his study. In contrast, chitosan oligomers with defined PAs by using different chemical and enzymatic methods have been reported [reviewed by Aam et al. 2010]. For example, hydrolytic enzymes for cleaving chitosan polymers [Mitsutomi et al., 1995; Sørbotten et al., 2005] or the deacetylation of oligomeric substrates by specific CDAs, like from *Vibrio cholerae* [Hamer et al., 2014], can be applied. Additionally, the production of a chitin oligomer was successful by reversing the enzyme function of ClCDA for acetylation, as shown for the tetramer AAAD [Tokuyasu et al., 2000b].

5.3.1. Biological activities of chitosans with non-random PA

Naturally occurring chitosans are created by the deacetylation of chitin by CDAs, being the only mechanism described apart from chemistry [Kaur and Dhillon, 2014]. As discussed above, the PA of a chitosan generated by a CDA may be random, blockwise for processive enzymes or possess a regular distribution of A and D units. Being fungal cell wall components, chitosans are biologically active in plant systems [Hadwiger, 2013], because they are naturally involved in pathogen-plant interactions [El Gueddari et al., 2002].

For plants, chitin/chitosan detection and adjusted signaling is important for the defense against pathogens [Kombrink et al., 2011] as well as for beneficial interactions towards rhizobia [Hamel and Beaudoin, 2010]. Further developing this thought, plant receptors evolved in the presence of natural chitosan and their PAs, hence, the application of random PA chitosan available might induce a different response than a non-random PA chitosan.

One reaction of plant cells towards chitosan as elicitor molecule is the production of reactive oxygen species [Boller and Felix, 2009]. For this reason, I used the oxidative burst assay to quantify the H_2O_2 production after the treatment of rice suspension-cultured cells to different chitosan samples [Ortmann et al., 2004; Paulert et al., 2010]. Because of a failure in sample preparation for the NMR measurements, most oxidative burst experiments were carried out using an enzymatically produced chitosan with a DA of 18% instead of 30%. The differences between PaCDA and chemically produced chitosan that were seen in these experiments (chapter 3.3.6, fig. 7B) can be explained by the lower DA of PaCDA chitosan. First, chitosan DA18% contains less GlcNAc units that can be recognized by the *O. sativa* chitin elicitor binding protein (CEBiP) [Kaku et al., 2006]. Second, the experiment with

PaCDA treated chitosan DA 29% created later did not show a clear difference to the solely-chemical chitosan (fig. 7A). In spite of this result concerning the biological activity, the proof of PA influencing the interactions between chitosans and biological systems might be demonstrated in further experiments using different activity assays.

The anti-microbial activity of chemical and enzymatic chitosan preparations towards *Fusarium graminearum* and *Lactobacillus plantarum* was investigated by Wattjes and revealed different MICs (minimal inhibitory concentrations) for both chitosans and species [Wattjes, 2014]. Although these results have to be confirmed using independently created and well-characterized chitosan batches, a higher growth inhibition for PaCDA deacetylated chitosan possessing a rather regular PA indicated that the evenly distributed positive charges might be the reason for this effect. By comparing different studies on anti-fungal, anti-bacterial and other biological activity assays, it has been proposed that the positive charge of the protonated GlcN amino group is responsible for interactions with cells [Aranaz et al., 2009]. Nevertheless, in many publications the chitin polymers and oligomers used were mixtures or not well-characterized leading to sometimes contradictory results on biological activities [Aam et al., 2010]. Hence, the use of chitosans with particular DP, DA and PA is an advantage for the development of chitosan-based product with defined biological activities.

5.4. Conclusions

Aiming for novel CDAs producing chitosans with non-random PA, good progress was made during the work for this thesis. For the first enzyme investigated, BC95, the protein production was challenging, nevertheless, successful in the yeast *H. polymorpha*, yet the CDA activity was not demonstrated. The production of BC95 in *E. coli* and *S. pombe* failed, and the expression of PaCDA in *E. coli* yielded only minimal protein amounts. But *H. polymorpha* has been proved to be a suitable host for the production of recombinant CDAs containing chitin binding domains by correctly expressing PaCDA. This enzyme was identified by comparing biochemical characterized CDA protein sequences to putative CDA sequences and then characterized being the first active CDA with CBDs until now. The analysis of the biochemical properties of PaCDA revealed similarities to the well-characterized ClCDA and suggested also a likewise, non-processive mode of action determined by subsite specificities. The activity of PaCDA towards crystalline substrates was not higher than other CDAs have been demonstrated earlier, thus the CBDs are not capable of loosening the chitin crystal. Three deletion constructs of

PaCDA lacking one or both CBDs were created to investigate the function of the CBDs for the enzyme, but only PaCDA-ΔN was successfully produced. Comparison of the two active CDAs to each other detected only minor differences between the single deletion variant and the full-length enzyme. By using PaCDA and a high DA chitosan (61%), a chitosan possessing a non-random PA was generated, which was demonstrated by enzymatic/mass spectrometric fingerprinting and ^{13}C-NMR analysis. Differing from the random PA of concurrently analyzed chemically produced chitosan with the same DP and DA, the alternate-dominated PA created by PaCDA in the recent study was the first described so far.

5.4.1. Perspectives

Further studies on CDAs containing CBDs could provide insights to the deacetylation of crystalline substrates and the process of generating chitosans with non-random PAs. Following the work presented in this thesis, five experiments for continuing these studies on BC95, PaCDA and chitosans with non-random PAs are suggested:

a) The optimization of BC95 production in *H. polymorpha* might result in active protein, which could then be further characterized. Additionally, experiments using different substrates other than chitin should be carried out to detect also other possible enzyme activities (chapter 2).

b) The mode of action of PaCDA might be unraveled further by using oligomers and investigating MS sequencing of intermediates and products, which might illustrate the subsite structure of PaCDA accordantly to ClCDA [Hekmat et al., 2003] (chapter 3).

c) The analysis of PaCDA crystal structure might yield valuable insights to the subsite structure and reaction mechanism of PaCDA similar to the study of ClCDA [Blair et al., 2006]. Furthermore the structures of the CBDs could be compared to each other and to the solved structures of CBM family 18 domains [Kezuka et al., 2010; Wright et al., 1991].

d) The construction of new C-terminal deletion constructs based on the results obtained from this study might reveal more information of the role the CBDs are playing for PaCDA in substrate binding, especially towards crystalline chitin, and mode of action (chapter 4).

e) The production of chitosan with non-random PA by PaCDA should be scaled up further to continue the analysis of physico-chemical properties and biological activities (chapter 3). These studies would ideally include as many CDAs as possible to compare different enzymatically produced PAs [Wattjes, 2014]

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