Chitin deacetylase: A comprehensive account on its role in nature and its biotechnological applications

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Chitin deacetylase (CDA; E.C. 3.5.1.41) is an enzyme that catalyzes the hydrolysis of acetamido groups of *N*-acetylglucosamine in chitin, promoting the conversion to chitosan, a glucosamine polymer. In zygomycetous fungi, CDA has an important role in fungal growth, being involved in cell-wall chitosan biosynthesis in tandem action with chitin synthase (CS; E.C. 2.4.1.16), and it could also be involved in deacetylation of chitin oligosaccharides during autolysis after the action of endo-chitinase on cell walls. The CDA is also reported to be involved in ascospore formation in *Saccharomyces cerevisiae*. An alternative biological role involving the enzyme in plant-pathogen interactions has been suggested for CDA from *Colletotrichum lindemuthianum*, a plant pathogen, and the enzyme is extracellular and active on chitin oligomers. The CDA and chitosanase facilitate the entry of the fungus, *Metarhizium anisopliae* in to the host insect, *Helicoverpa armigera*. In the case of plant pathogens such as *Uromyces viciae-fabae*, and an insect pathogen, *M. anisopliae* chitin deacetylase is known to assist in self defense from host chitinases by changing the wall composition from chitin to chitosan. The purification and characterization of CDA and identification and characterization of CDA genes from different organisms have also been reported.

Chitosan, a deacetylated product of chitin, is a natural polymer that has great potential in biotechnology and in the biomedical and pharmaceutical industries. Commercially, it is produced from chitin *via* a harsh thermo-chemical process that shares most of the disadvantages of a multi-step chemical procedure. It is environmentally unsafe and not easily controlled, leading to a broad and heterogeneous range of products. An alternative or complementary procedure exploiting the enzymatic deacetylation of chitin could potentially be employed, especially when a controlled and well-defined process is required. The different biotechnological applications of CDA will be discussed.

Keywords: Chitin deacetylase, chitosan, roles and applications of chitin deacetylase

1. Introduction

Chitin is known from ancient times and has been found in the shell of 100 megayear old Pterygotus (ancient scorpion). Henri Braccanot in 1811 discovered this polymer from the fungal species of *Agaricus, Hydnum* and *Boletus* and called it “Fungine”. In 1823, A. Odier found this polymer in insect cuticle and named it chitin - a Greek term for tunic / envelope. Chitin is a polymer of *N*-acetyl-D-glucosamine with β-1, 4 linkage similar to cellulose. In nature, 10 gigatons of chitin is synthesized and degraded each year. The chitin polymer displays 0.9% degree of acetylation, 7% nitrogen content and nitrogen/carbon ratio of 0.146 [1]. Chitin is not readily soluble and that limits its industrial applications [2]. The deacetylated form of chitin is called chitosan and is relatively soluble. The degree of acetylation is less than 0.4% and nitrogen content >7% [3]. Natural synthesis of chitosan occurs as an abundant wall component of zygomycetous fungi (Chart 1).

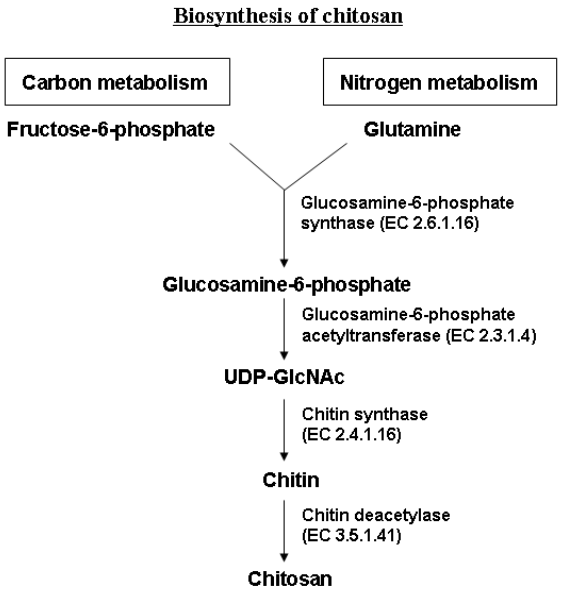

Chart 1. Synthesis of chitosan in nature

Chitosan has a great potential in biotechnology and in the biomedical and pharmaceutical industries [4]. Commercially, it is produced from shrimp/ crab/ squid chitin *via* a harsh thermo-chemical process that shares most of the disadvantages of a multi-step chemical procedure. It is environmentally unsafe and not easily controlled, leading to a broad and heterogenous range of products. An alternative or complementary procedure exploiting the enzymatic deacetylation of chitin could potentially be employed, especially when a controlled and well-defined process is required.

Chitin deacetylase (CDA, EC 3.5.1.41) is an enzyme that catalyzes the hydrolysis of acetamido groups of *N* - acetylglucosamine (GlcNAc) in chitin, promoting the conversion to chitosan, a glucosamine polymer (Chart 1). Deacetylation of chitin by CDA provides an environmentally safe enzymic process to convert the crustacean / fungal chitin into chitosan resulting in a more homogenous product for a variety of applications. CDA from zygomycetous and non- zygomycetous fungi have been characterized at the biochemical and molecular level. The biochemical characterization of CDA from different organisms offers insight for monitoring its potential for up scaling at the commercial level. Molecular cloning of CDA has been attempted to enhance the production and recovery of the enzyme in its active state for commercial applications.

2. Biochemical characteristics of chitin deacetylases

In general, chitin deacetylase has been isolated and purified from mycelium extracts of fungi, such as *Mucor rouxii* [5] , *Absidia coerulea* [6] and *Aspergillus nidulans* [7] and from the culture filtrate of the fungus *Colletotrichum lindemuthianum* [8, 9] (Table 1). Extracellular CDA from *Scopulariopsis brevicaulis* has been purified and characterized [10] (Table 1). Mycelial extracts from *Cunninghamella bertholletiae* [11] has been partially purified (Table 1). Chitin deacetylases are produced by fungi either in the periplasmic space or extracellularly depending on their function. CDA from *M. rouxii, A. coerulea*, *A. nidulans* and *C. bertholletiae,* secreted into the periplasmic space are generally associated with cell wall modification (Table 1). Extracellular CDA secreted into the culture medium was reported to be associated with mycelial growth and autolysis in case of *M. anisopliae* and *C. lindemuthianum* while it was found to be secreted during autolysis in *A. nidulans* [7, 8, 12]. Extracellular CDA activities from *C. lindemuthianum* and *M. anisopliae* were suggested to possess dual function in modification of chitin as well as chito-oligomers for the interaction with host and in self- defense.

Table 1 Biochemical characteristics of fungal chitin deacetylases

Organism	Optimum		pI	Acetate inhibition	Metal ion effect	Isozymes	Mol. Mass (KDa)
	Temp. (°C)	pH					
*A. coerulea**	50	5.0	NA	Yes	+ (Mn^{2+})/ - (Fe^{2+})	NA	75
*A. nidulans**	50	7.0	2.75	No	- (Cd^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Sn^{2+}, Mg^{2+}, Mn^{2+}, Ag^{2+}, Pb^{2+})	2	27.5 & 27.3
*B. poitrasii**	50	NA	NA	NA	NA	NA	NA
*C. lindemuthianum***	60	11.5	3.7	No	+ (Co^{2+})/ - (Co^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+})	NA	31.5-33
*C. lindemuthianum***	NA	8.5	NA	No	NA	NA	150
*C. lindemuthianum***	50	8.5	3-5	No	+ (Co^{2+}) - (Zn^{2+}, Mn^{2+}, Na^{2+})	NA	<150
*C. lindemuthianum***	60	8.0	NA	NA	+ Co^{2+} - Mg^{2+}, Ca^{2+}, Fe^{2+} Cu^{2+}, Ni^{2+}, Zn^{2+}	NA	25
*C. berthollitae**	50	4.5	NA	NA	NA	NA	NA
*F. velutipes**	60	7.0	NA	No	+ (Co^{2+}, Ca^{2+}, Zn^{2+} - (Cu^{2+}, Ni^{2+})	NA	31
*G. butleri**	NA	NA	NA	NA	NA	NA	70
*M. anisopliae***	30	8.5	2.65, 3.8, 4.11	No	NA	3	70, 37, 26
*Mortierella sp.**	60	5.5	NA	Yes	+ (Ca^{2+}, Co^{2+}) - (Hg^{2+}, Zn^{2+}, Ag^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Sn^{2+})	2	50 & 59
*M. rouxii**	NA	5.5	3.0	Yes	+ (Zn^{2+}) /- (Co^{2+} Mn^{2+}, Na^{2+})	NA	75
*M. rouxii**	50	4.5	3.0	NA	NA	NA	75-80
*M. racemosus**	NA	7.0	NA	NA	NA	3	26-64
*R. circinans**	37	5.5-6.0	NA	NA	+ (Mn^{2+}, Mg^{2+}) - (Cu^{2+})	NA	75
*R. nigricans**	NA	7.0	NA	NA	NA	4	26-64
*R. nigricans**	NA	NA	NA	NA	NA	NA	100

*S. cerevisiae**	50	8.0	NA	Yes	+ (Co^{2+})/ - (Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}	NA	43
*S. brevicaulis***	55	7.5	NA	NA	NA	NA	55
*U. viciae-fabae***	NA	5.5-6.0	NA	NA	NA	5	48.1, 30.7 25.2, 15.2, 12.7

* : Periplasmic CDA ; ** : Extracellular CDA.

References: [6-13, 15, 16, 19, 27, 29, 38, 40, 80-85]

CDA of periplasmic origin is generally inhibited by acetate (Table 1). While extracellular CDAs generally do not show any change in activity in presence of acetate however *A. nidulans* CDA showed activation in the presence of acetate (0.4-4mM) [7]. CDA from *M. anisopliae* was seen to exhibit activity in presence of the inhibitors such as acetate and melanin. Melanin inhibited the activity of *M. anisopliae* chitinases but did not affect the CDA activity [12].

Extracellular CDA from *M. anisopliae*, secreted into the culture medium, showed activity in the pH range 7.5–9.2, and the optimum pH for the enzyme activity was found to be 8.5 [12]. In case of CDA secreted into culture medium of *C. lindemuthianum* and *S. brevicaulis* the optimum pH exhibited was also high (pH 11.5 and 7.5, respectively). Lower pH optimum (pH 5.5) was reported for periplasmic CDA from *Benjaminiella poitrasii* [13], *M. rouxii*, *A. coerulea*, *A. nidulans*, *S. cerevisiae*, *Rhizopus nigricans* and *C. bertholletiae* (Table 1). The proportion of chitosan in yeast form was found to be more than that of mycelial form in the dimorphic fungus *B. poitrasii* and acidic pH favoured yeast form. It could be interesting to find biochemical correlation of CDA activity with the morphological outcome [14].

The temperature optima above 50°C were reported for CDA from *A. nidulans, C. lindemuthianum, S. brevicaulis* and *B. poitrasii*. In case of *A. nidulans* though the optimum temperature of the deacetylation reaction was 50˚C, the enzyme was found to be stable at 100˚C for 1h [7].

The pI value for CDA from *C. lindemuthianum* was 3.7 and 3.0 for *M. rouxii*. The isoelectric focusing of the ammonium sulphate precipitated fraction (65–80%) of the *M. anisopliae* culture filtrate showed activity at pH 2.65, 3.8, and 4.11 [12].

Upto five isozymes of CDA were reported in several fungi (Table 1). In the case of *Uromyces viciae-fabae*, the broad bean rust fungus, five isozymes of CDA were reported in coordination with penetration of the fungus through the leaf stomata [15]. The apparent molecular masses of the five CDA isozymes ranged from 12.7 to 48.1 kDa. Trudel and Asselin [16] reported three isozymes of CDA in *M. racemosus* and four isozymes in *R. nigricans* with molecular masses ranging from 26- 65 kDa. The purified *C. lindemuthianum* CDA, was between 31.5 and 33 kDa, while in case of *M. rouxii* molecular mass was 75 kDa [5, 9]. Three isozymes have been reported for *M. anisopliae* with apparent molecular masses of 70, 37, and 26 kDa [12]. Recently, Wang et al [17] PCR amplified, cloned and expressed chitin deacetylase from *A. nidulans* in *Escherichia coli* and found that the molecular mass of the expressed protein was 24.2 kDa unlike the previous CDA (19.5 kDa) reported from *A. nidulans* [7]. The two CDAs had different Km and optimal pH suggesting the presence of isozymes in *A. nidulans.*

Metal ions are known to act as activators or inhibitors of enzyme activity. The metal ions such as Co^{2+}, Mn^{2+}, Na^{+} or EDTA may act either as activators or inhibitors for CDA depending on their concentration. For instance, Zn^{2+} (1mM) slightly promoted the CDA activity in *C. lindemuthianum* but, increase in concentration to 10mM strongly inhibited activity of the enzyme [9]. In case of *S. cerevisiae* and *C. lindemuthianum* CDA was activated in presence of Co^{2+} but inhibition occurred in case of CDA from *A. nidulans* (Table 1). The different source of CDA and the varying concentration of metal ions used could be the possible reason for the different response to the presence of metal ions. Blair et al [18], recently deduced structure of *C. lindemuthianum* CDA by mass spectroscopy and suggested that the substrate binding region consisted of a conserved histidine-histidine-aspartic acid zinc binding triad that carried out the acid/base catalysis. They proposed that *C. lindemuthianum* CDA was a metalloenzyme that showed alteration in enzyme activity in response to subtle alterations in substrate specificity and sub-site affinity.

Different substrates with different length and degree of deacetylation were used to study the specificity of CDA. For instance, *M. rouxii* and *C. lindemuthianum* had maximum affinity towards the substrates $(GlcNAc)_{4\text{-}6}$ [5,9]. In case of *M. anisopliae* CDA enzyme, the highest activity was detected with ethylene glycol chitin [12].

The mode of action of CDA from *M. rouxii* on chitin oligosaccharides (DP or degree of polymerization 2-7) was studied [5, 19]. The enzyme could not effectively deacetylate chitin oligomers with a degree of polymerization lower than three. Tetra-*N*-acetylchitotetraose and penta-*N*-acetyl chitopentaose were fully deacetylated by the enzyme, however, in tri-*N*- acetylchitoheptaose, the reducing end residue remained intact. It was suggested that CDA needed minimum $(GlcNAc)_3$ - $(GlcNAc)_4$ consecutive residues for the activity [5, 7, 20].

Chitin oligomer of sufficient length (degree of polymerization higher than 2) is required for the enzyme to act on the substrate. The formation of the enzyme – substrate complex in case of *M. rouxii* induced the deacetylation that took place at the non-reducing end residue of the oligomer [21]. Following the first deacetylation, the enzyme catalysed sequentially the hydrolysis of the following acetamido groups before it was dissociated and formed a new active complex with another chitin oligomer. In case of $(GlcNAc)_4$ and $(GlcNAc)_5$, the geometry of the enzyme – substrate complex favoured the complete deacetylation of the substrate, while for shorter or longer oligomers the reducing – end residue remained intact [22].

3. Demonstration of chitin deacetylase activity

Different substrates with different degrees of deacetylation were used to measure CDA activity. These include glycol chitin, colloidal chitin, powdered chitin, practical grade chitin (BDH), crab chitin, Katakura chitin, Sigma α chitin, chitin CHA-2, γ-chitin, colloidal chitin, carboxymethylchitin, α-1→3,1→6-*N*-acetylgalactosamine galactan, *N*-acetylglucosamine and $(GlcNAc)_{2\text{-}6}$ [5, 7, 9, 12, 19].

A radioactive assay employing O-hydroxyethylchitin (glycol chitin), radiolabelled in the *N*-acetyl group, as substrate was reported for the determination of chitin deacetylase activity in *M. rouxii* [23]. The method was sensitive, though evaluation of the extent and distribution of derivatization (O-hydroxyethyl groups) in commercial glycol chitin and the effect of derivatization on enzyme activity was difficult to determine. Furthermore, this method could not be used for monitoring deacetylation processes of non-radiolabelled natural substrates. Kauss and Bausch [24] used nitrous acid and 3-methyl-2-benzothiazoline hydrazone (MBTH) for depolymerization of the ethylene glycol chitin with $NaNO_2$ and colorimetric detection of glucosamine released with MBTH. CDA activity was also measured with acetate released from hexa-*N*-acetylchitohexaose using the enzymatic method of Bergmeyer via three coupled enzyme reactions [25].

The isozymes of CDA were detected by activity staining method of Trudel and Asselin [16] involving transfer of enzymes from PAGE gel by diffusion to ethylene glycol containing gel. Differential staining of native PAGE using a fluorescent brightener (Calcofluor white M2R) was investigated. Calcofluor white M2R interacted more strongly with chitosan than with chitin embedded in the gel and was much more fluorescent under UV even after prolonged destaining. Furthermore, the CDA activity can also be detected by depolymerization of chitosan by nitrous acid which makes the same band non-fluorescent. Recently, Toprak et al [26] showed that protein transfer by electroblotting ensured quick transfer of CDA onto the chitin containing gel for activity staining.

4. Molecular characteristics of chitin deacetylases

Multiple sequence analysis revealed that similar to chitinases the CDA genes from different organisms also do not share a common amino or carboxyl terminal. CDA genes from different fungi were reported to share conserved amino acids in the polysaccharide deacetylase domain lying in the middle of the genes [27-30]. The CDA sequences shared maximum homology between *G. butleri* and *M. rouxii* (48% identity) followed by *Phycomyces blakesleeanus* (30% identity) [29]. On the other hand 14 % and 12% identity were observed with *Blumeria graminis* and *C. lindemuthianum* CDAs [29].

Conserved polysaccharide deacetylase domain analysis revealed a 140-147 aa residue functional domain encoded by the middle part of the gene sharing a high similarity with CDAs of fungi and bacterial peptidoglycan deacetylases. Furthermore, nod-B like protein from *Sinorhizobium meliloti, Bacillus subtilis*, xylanase D from *Cellulomonas fimi* and acetylxylan esterase A from *Streptomyces lividans* also showed same functional domain [27-29, 31, 32]. Numerous conserved residues were observed that possibly could play a common role in functional catalytic reactions of deacetylases. No other domain as a substrate-binding domain was reported to be encoded, suggesting that the enzyme is involved primarily in catalyzing the deacetylation of chitin [28]. Recently resolved structure and function of *C. lindemuthianum* CDA protein adopted a $(\beta/\alpha)_8$ fold similar to other glycohydrolases, with a putative substrate binding site harbouring a majority of the conserved amino acid residues [18].

The first CDA gene isolated, characterized and sequenced was from *M. rouxii*. [28]. In *S. cerevisiae* two genes *CDA1* and *CDA2* were identified and sequenced by homology comparison to *M. rouxii* CDA gene from the cosmid library constructed for the yeast [31, 32]. In *C. lindemuthianum*, CDA gene was isolated using primers designed towards the conserved central region of the CDA gene that showed maximum homology to CDA genes from other fungi. This gene was characterized and sequenced and later overexpressed in *E.coli* [30, 33]. CDA genes from *G. butleri*, *R. nigricans* and dimorphic human pathogens *Cryptococcus neoformans* and *Paracoccoidioides brasiliensis* were also reported [27, 29, 34, 35].

5. Biological roles of CDA

5.1 Cell wall formation in fungi

Relatively more chitosan to chitin ratios in the cell wall of zygomycetous fungi like *A. coerulea, M. rouxii , B. poitrasii, Rhizopus delemar, Cunnighamella blackesleeana* and *Mortierella isabelina* and others were reported. According to the model proposed by Bartnicki-Garcia for the biosynthesis of chitin and chitosan, nascent chitin chains are modified by CDAs during their synthesis by the enzyme chitin synthase [36]. The synergistic action of chitin synthase – chitin deacetylase resulted in chitin - chitosan containing fibrils that crystallized to form the main structural mesh of the cell walls. CDA enzyme could also be involved in deacetylation of chitin oligosaccharides during autolysis after action of endo-chitinases on the cell wall.

5.2 Ascospore wall formation in yeasts

Chitosan is found in the ascospores of *S. cerevisiae* and *Schizosaccharomyces pombe* [37, 38]. The role of chitin deacetylases in the formation of the *S. cerevisiae* spore walls was investigated by comparison of the dityrosine and glucosamine contents of spore walls from a wild type strain, *CDA1* and *CDA2* disruption strains and a double disruption strain. Detection of low levels of dityrosine was associated with highly acetylated chitin and the decrease of deacetylation affected the total quantity of the chitin polymer in the wall, making the ascospore more susceptible to hydrolytic attack and dessication. Earlier, it was shown that the outermost layer of the yeast spore wall consisted of a dityrosine-rich polymer closely associated with the underlying chitosan layer. Especially in the absence of Cda2 protein (Cda2p) it appeared that the remaining chitin could not form a distinct polymer. Elimination of all CDA activity prevented any addition to this layer. Consequently, it was demonstrated that not only chitosan but chitin synthesis was also dependent on chitin deacetylation as decrease in deacetylation affected the quantity of chitin produced [39]. In the fission yeast *S. pombe* it was suggested that the roles of chitin as well as chitosan were limited to spore formation process only [38].

5.3 Appressorium formation in plant pathogens

Chitin deacetylase was reported to play an important role in plant–pathogen interactions [8, 40]. For instance, CDA deacetylates the cell wall chitin of the pathogen for the self-defense against plant endochitinases. In *Magnaporthe grisea*, *C. lindemuthianum* and several rust fungi, the cell walls showed decreased affinity to wheat germ agglutinin (WGA), suggesting either lowered chitin content or a modification of chitin [41-43]. In *U. viciae-fabae*, an increase in CDA activity was observed during appressorium development [15].

Recently, it was reported that *M. grisea* CDA could possibly be involved in sensing the factors that induced appressorium formation. For instance, CDA deficient mutants were unable to form appressoria on artificial surface such as polycarbonate. Since the null mutant Cbp1 (chitin binding protein) showed abnormal appressorium differentiation only on artificial surfaces and was sensitive to the chemical inducers, *CBP1* gene expression suggested to play an important role in the recognition of physical factors on solid surfaces [44].

5.4 The fungus-insect interaction

The insect-pathogenic fungi act by contact to initiate killing process of the insect [45]. Entomopathogenic fungi generally invade the insect host by a combination of mechanical pressure and secretion of cuticle degrading enzymes. For penetration through the insect cuticle, fungi like *Metarhizium* and others produce cuticle degrading enzymes such as chitinases, proteases and lipases [46, 47]. It was reported that melanization of the cuticle made insect host comparatively resistant to proteolytic and chitinolytic enzyme attack [48, 49]. It was reported that *M. anisopliae* strains constitutively produced extracellular CDA which converted insect cuticular chitin into chitosan facilitating the entry of the fungus into the insect body [12, 47]. The same was reported to play a significant role in self-defense from the insect chitinases produced during moulting process [12].

5.5 CDA and human pathogens

Cell-mediated immunity plays a crucial role in host defenses against the dimorphic human pathogenic fungus *C. neoformans,* causal agent of cryptococcosis [34]. For the first time, a 25-kDa extracellular polysaccharide deacetylase, was shown to induce protective immune responses.

In case of *P. brasiliensis*, the causative agent of paracoccidioidomycoses, a disease that affected 10 million people in Latin America the transcriptional analysis identified a CDA gene that was upregulated in the pathogenic yeast form of the fungus [35].

The human pathogen *Encephalitozoon cuniculi,* is a microsporidial fungal-like unicellular obligate intracellular parasite. The presence of chitosan in the microsporidian spore wall was accounted for the extreme rigidity and

resistance of the parasite to chitinase treatments [50]. During the spore development, TEM studies detected accumulation of the enzyme especially in paramural bodies which are convolutions of the plasma membrane opened to the wall. It was suggested that chitin might be synthesized and deacetylated within the paramural body before being incorporated to the wall in a process similar to the fungal chitosomes [50]. The cyst wall of *Entamoeba invadens* was shown to contain substantial quantities of chitosan, consistent with the presence of a functional chitin deacetylase [51].

5.6 Bacterial deacetylases

Number of marine bacteria widely distributed in oceanic and estuarine waters are mainly responsible for recycling of nitrogen present in chitinous debris. Earlier it was shown that chitin hydrolysis was carried out by atleast two enzymes, a chitinase that mainly produced *N, N′*-diacetylchitobiose $(GlcNAc)_2$, and a beta-*N*-acetylglucosaminidase that gave the final product, GlcNAc [52]. Recently Jung et al, [53] described the involvement of CDA genes in the chitin catabolic cascade of Vibrios. Microarray expression profiling and mutational studies of *Vibrio cholerae* showed that chitobiose induced genes were required for the transport and catabolism of non-acetylated chitin residues [54]. Li et al [55] purified *V. cholerae* CDA and demonstrated that it was very active with chitin oligosaccharides that were converted to products lacking one acetyl group, because it hydrolyzed the *N*-acetyl group attached to the penultimate GlcNAc unit. The gene bank sequence data showed that CDA was highly conserved in Vibrios and Photobacteria. The GlcNAc-$GlcNH_2$- $(GlcNAc)_n$ products of CDA action, resembled those obtained by hydrolysis of the chitooligosaccharides with Nod B: $GlcNH_2$-$(GlcNAc)_{3–4}$ involved in the biosynthesis of Nod factors. Furthermore, it was suggested that the oligomers generated by deacetylases could play a role in cellular communications.

Fungal CDAs have shown evolutionary relatedness with nodB protein of rhizobial origin [28]. Recently Maw et al, [29] demonstrated the phylogenetic relatedness of the conserved central domain of the CDA gene with the nodB factor of *S. meliloti*. Rhizobia are known to interact with their leguminous host plants e.g. *S. meliloti* interacts with *M. sativa* to establish nitrogen fixing symbiosis [56]. Legume roots secrete nodulation signals called Nod factors which are lipo-chitooligosaccharides i.e. β-1, 4 linked oligomers of GlcNAc, with a fatty acid replacing the *N*-acetyl group on their non-reducing ends [57, 58]. On the basis of the similarity of nodB protein to chitin deacetylase John et al [59] suggested that the specific deacetylation of the non-reducing end of the precursors provide the necessary free amino group for a subsequent acylation.

5.7 Insect chitin deacetylases

Previously, though most of the CDAs were reported from fungi and bacteria recent studies show their presence in arthropods as well [60, 61]. In *Drosophila melanogaster* proteins with CDA domains in tracheae restricted the elongation of tracheal tubes, presumably by modification of terminal GlcNAc of the elongating chitin chain [60, 62]. Dixit et al [61] reported the presence of 5 major classes of CDA like proteins in *Tribolium castaneum*. One of them was specifically expressed in the gut. Similar type of proteins were also reported in other insect species such as *Anopheles gambiae, Apis mellifera , Bombyx mori, D. melanogaster*, *Epiphyas postvittana, Helicoverpa armigera , Mamestra configurata , Spodoptera frugiperda* and *Trichoplusia ni* [26, 60-68]. Most of the reported insect CDAs and/or CDA like proteins are associated with the midgut peritrophic membrane (PM) and evenly distributed throughout the entire length of PM. Presence of these enzymes in the midgut tissue of larvae was detected only during the feeding period that may be associated with increased absorption of nutrients. In *M. configurata* CDA activity was detected from gut proteins expressed in *E. coli* [26].

6. Evolutionary significance of chitin deacetylase

Maw et al [29] carried out a phylogenetic analysis of CDAs of different fungi. According to them, the evolutionary relationship was class specific in fungi. It has been shown that fungal CDAs are homologous to nodulation enzymes and peptidoglycan deacetylases present in gram positive bacteria [28]. Homologous enzymes were reported to be absent in plants, animals, and most protists (e.g. *Plasmodium*, *Dictyostelium*, *Trypanosoma*, and *Giardia*) with the exception of *Entamoeba* and *Trichomonas* [51]. Homologous deacetylases were also absent from archaea with the exception of *Methanosarcina*. It has been suggested that the scattered distribution of the deacetylases may be from extensive secondary loss, if a common ancestor contained the gene encoding the enzyme. Alternately, this distribution may result from a series of lateral gene transfers (LGTs), which may not respect normal phylogenetic groupings.

A phylogenetic reconstruction using the maximum likelihood method of bacterial and eukaryotic chitin/ polysaccharide deacetylase was star-shaped, so it was difficult to make strong inferences concerning the origins of the deacetylase genes [51]. The results suggested the possibility of LGT rather than secondary loss. First, the eukaryotes and prokaroyotes were not clearly separated in the phylogenetic tree. If the common ancestor to each contained a deacetylase gene, eukaryotes and prokaryotes should form distinct clades. Second, *Entamoeba* and *Trichomonas* enzymes were in separate clades, and fungal enzymes were in separate clades. Third, there was a good bootstrap support

(81%) for the inference of LGT between *Trichomonas* and *Bacteroides thetaiotaomicron*. While chitin deacetylases were present in numerous fungi and bacteria, putative chitin deacetylases of *Entamoeba* and *Trichomonas* were the first identified in protists [51].

7. Applications of chitin deacetylases

Chitosan is produced from chitin and the two polymers are differentiated on the basis of their degree of deacetylation. These polymers and their derivatives are currently in demand worldwide. The various uses of chitosan are illustrated in Table 2.

Table 2. Applications of chitosan

Application	Reference
Food industry	
• Animal feed additives	[86]
• Food preservation	[87]
Biomedical applications	
• Wound dressing	[88]
• Dietary supplement	[89, 90]
• Drug delivery and therapies	[91-94]
• Dentistry	[102]
Cosmetic applications	[95]
Industrial applications	
• Immobilization of enzymes	[96]
• Use in ethanol sensor	[97]
Membranes	[98]
Water engineering	
• Metal capture from wastewater	[99, 100]
• Colour removal from textile mill effluents	[101]

Presently, chitosan is being isolated from crab and shrimp shells by a thermo- chemical process. However it has some drawbacks like use of concentrated alkaline solution, resulting in an increase in the level of environmental pollution and heterogenous product formation. In order to overcome these limitations, naturally occurring chitosan from certain Zygomycetes fungi can be isolated from their mycelial biomass. Some fungi possess a low molecular weight chitosan that is useful for medical applications and in agriculture [13]. As insoluble crystalline chitin is resistant to enzymatic deacetylation, it is first deacetylated partially by chemical treatment and is further deacetylated by CDA while the soluble chitooligomers, are more efficiently deacetylated by CDAs. The enzymatic deacetylation is not a random process like chemical deacetylation. Therefore, new chitosan oligomers with well-defined range of molecular weight and the content of *N*-acetylated residues can be produced.

C. lindemuthianum CDA was used to synthesize a nitrophenol labeled novel substrate, p-nitrophenyl- 2 amido-4-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (GlcNGlcNAc-pNP) to differentiate the action of *N*-acetylglucoaminidases. Selective N- deacetylation of the acetamido sugar to amido sugar residue at the non-reducing end with CDA was used to generate the unique substrate [69]. This substrate was used to distinguish between the action of *N*-acetylglucoaminidases that hydrolyse the substrate in a stepwise manner and those that degrade the substrate by recognizing the terminal residue [69].

A. coerulea CDA was immobilized for the first time by Jaworska et al [70] on cellulose-based Granocel matrix (DEAE- Granocel) activated with divinyl sulfone which offered the highest activity and stability by covalent binding of chitin deacetylase. *A. coerulea* CDA plays an important role in enzymatic deacetylation of chitosan to obtain polymers with a lower degree of acetylation.

Chitosan content of zygomycetous fungi like *A. coerulea, R. delemar, C. blackesleeana, M. rouxii*, *B. poitrasii* and *M. isabelina,* range between 6.1-11% of the dry weight of cells [13, 71, 72]. Fungi of this group can be readily grown in

the laboratory on cheap nutrients and the wall material can be recovered by simple chemical procedures [73]. Therefore chitosan can be produced in a controlled environment all year round and be independent of seasonal shellfish industry [74, 75]. Enzymic conversion of chitin to chitosan is another method to avoid the environmental concerns due to the chemical conversion. *Aspergillus niger* waste mycelium from citric acid production plant was efficiently converted to chitosan employing the extracellular CDA from *S. brevicaulis* [10].

Further, with the help of molecular biology techniques, the CDAs can be manipulated to get enzymes with novel properties which can be used for the preparation of chitosan polymers and oligomers. CDA from *C. lindeuthianum* was expressed in *E.coli* however recovery of the enzyme from the inclusion bodies showed low activity [33]. Recombinant CDA was produced in the culture media of *E.coli* in a highly active form using the signal sequence of *Streptomyces lividans* [30, 69]. Cloning and over-expression of chitin deacetylase gene has application in large-scale production of the enzyme for its commercial use. In *A. nidulans* CDA expression in *E. coli* for industrial application was not convincing [17]. Other eukaryotic expression systems such as the yeast *Pichia pastoris* may be useful for expression of CDA. The recombinant chitin deacetylase from *C. lindemuthianum* expressed in *Pichia pastoris* was significantly activated by Co^{2+} ions [17].

The cell wall provides a target for antifungal therapy as it contains chitin which is absent in humans. The chitosan produced by the CDA treatment of nascent chitin is an important component of the protective covers of pathogens [50, 51, 76]. The inhibition of CDA could also support the fungal cell wall hydrolysis by plant chitinases, thus the control of the plant pathogenic fungi becomes feasible [9].

Similarly, CDA could also be a versatile tool in the biological control of insect pests. In the insect pathogenic fungus *M. anisopliae*, CDA proved to be important in initiating pathogenesis by softening the insect cuticle to aid mycelial penetration. CDA herein may have a dual role in modifying the insect cuticular chitin as well as in altering its own cell walls for defense from insect chitinase [12]. CDA could be used in combination with other hydrolytic enzymes for control of pests and pathogens [12]. In plant pathogenic fungus *C. lindemuthianum* as Cu^{2+} was inhibitory to CDA [9], fungicides containing copper could be effectively used for the pathogen control. CDA from midgut of *Helicoverpa armigera* was reported to be downregulated in response to Baculovirus infection reducing the peritrophic membrane permeability [77]. This was attributed to a possible mechanism to reduce susceptibility to Baculovirus. This mechanism was used for the biocontrol by engineering recombinant nucleopolyhedrosis virus to express CDA [77].

In the biological control of pest insects, chitin deacetylase proved to be a potential target for an insecticide. Chitin deacetylase is a major protein secreted in the peritrophic matrix of the arthropod gut during feeding; it can modify the chitin component in such a way as to protect the gut from parasite invasion, and intercept toxins like lectins [64]. Thus, the inhibition of this enzyme represents a potential way to control the pest insects.

The marine bacterium *Vibrio vulnificus* causes highly lethal sepsis and destructive wound infections with severely rapid pathological progress in shellfish. Lee et al [78] showed that partially depolymerized chitosan prevented and treated infection generated by *V. vulnificus*.

One of the interesting applications of CDA could be in transgenics. Genetic manipulation of plants can be carried out by expression of chitin and chitosan in plants to alter the cell wall for industrial uses and improved disease resistance. Recently, there has been interest in the paper and textile industries in developing chitin:chitosan and chitosan:cellulose blends. Chitin and chitosan can be produced in plants by expressing chitin synthase and CDA genes in plants so that such transgenic plants can be used as a single source of cellulose, chitin and chitosan [79].

Chitosan is always formed by the modification of chitin which makes the role of chitin deacetylase thus important in biology as well in the commercial use of the polymer. The enzyme itself has lot of potential for its direct use for the synthesis of novel oligosaccharides and in integrated pest management [103] .

Acknowledgement: The authors thank the Department of Biotechnology, New Delhi and the Swiss Agency for Development and Cooperation, Berne, Switzerland for financial support.

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