Purification and Characterization of Chitin Deacetylase from Colletotrichum lindemuthianum*

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Chitin deacetylase (EC 3.5.1.41), the enzyme that catalyzes the hydrolysis of acetamido groups of N-acetyl-\(\beta\)-(1→4)-glucosamine in chitin, has been purified to homogeneity from the culture filtrate of the fungus Colletotrichum lindemuthianum and further characterized. The enzyme is a glycoprotein, and its apparent molecular mass was determined to be ~150 kDa. The glycosylation pattern of the enzyme is consistent with a mixture of N-linked glycans including oligomannosidic hybrid and/or complex type, and its carbohydrate content is approximately 67% by weight. Chitin deacetylase is active on several chitinous substrates and chitin derivatives, is not considerably inhibited by carboxylic acids, especially acetic acid, and exhibits a remarkable thermostability. The enzyme requires at least two N-acetyl-\(\beta\)-(1→4)-glucosamine residues (chitobiose) for catalysis. When glycol chitin (a water-soluble chitin derivative) was used as substrate, the optimum temperature for enzyme activity was determined to be 50 °C, and the optimum pH was ~8.5.

Chitin, a homopolymer of \(\beta\)-(1→4)-linked N-acetyl-\(\beta\)-D-glucosamine, is one of the most abundant, easily obtained, and renewable natural polymers, second only to cellulose. It is commonly found in the exoskeletons or cuticles of many invertebrates (1) and in the cell walls of most fungi and algae (2).

Chitin is an extremely insoluble material and has yet to find an important industrial use, whereas chitosan (the deacetylated form of chitin) is water-soluble and a much more tractable material with a large number and a broad variety of reported applications (3–5). At present, chitosan is produced by the thermochemical deacetylation of chitin. An alternative or complementary procedure exploiting the enzymatic deacetylation of chitin could potentially be employed, especially when a controlled, non-degradative, and well defined process is required.

Chitin deacetylase (CDA),\(^1\) the enzyme that catalyzes the conversion of chitin to chitosan by the deacetylation of N-acetyl-\(\beta\)-(1→4)-glucosamine residues, was first identified and partially purified from extracts of the fungus Mucor rouxii (6). Since then, the presence of this enzyme activity has been reported in several other fungi (7–9) and in some insect species (10). The purification of the first chitin deacetylase to homogeneity and further characterization of the enzyme from the fungus M. rouxii has been recently reported. The enzyme is an acidic glycoprotein of ~75 kDa with a carbohydrate content of ~30% by weight. It exhibits a very stringent specificity for \(\beta\)-(1→4)-linked N-acetyl-\(\beta\)-D-glucosamine homopolymers, requires at least four residues (tetra-N-acetylchitotetraose) for catalysis, and is inhibited by carboxylic acids, particularly acetic acid. The optimum temperature for enzyme activity was determined to be ~50 °C and the optimum pH ~4.5. A cDNA of the M. rouxii encoding CDA was isolated, sequenced, and further characterized (13). Protein sequence comparisons revealed significant similarities between the fungal chitin deacetylase and the rhizobial nodB proteins, suggesting functional homology of these evolutionarily distant proteins. The functional assignment of the nodB protein in Nod factor biosynthesis, as deduced from its sequence similarity, was subsequently verified biochemically (14). The purification and partial characterization of chitin deacetylasdes from Absidia coerulea (15) and Aspergillus nidulans (16) has also been recently reported. CDA from A. coerulea as compared to the M. rouxii enzyme exhibited similar molecular weight, amino-terminal sequence, pH and temperature optimum, and substrate specificity. However, CDA from A. nidulans as compared to the above enzymes exhibited different molecular weight, pH optimum, and substrate specificity.

Initiating a study to elucidate the potential biological role of CDA activity and further evaluate the potential use of an enzymatic process for deacetylation of chitin substrates, we now report the isolation and characterization of CDA from the fungus Colletotrichum lindemuthianum.

EXPERIMENTAL PROCEDURES

Materials—C. lindemuthianum (DSM, no. 63144) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Glycol chitosan, concanavalin A (type IV), and N-acetylchitooligosaccharides (dimer to tetramer) were purchased from Sigma. N-Acetylchitooligosaccharides (pentamer and hexamer) were purchased from Accurate Chemical (Westbury, NY). \(^{1}\)\(^{1}\)H\)Acetic anhydride was obtained from DuPont NEN. Enzymes and reagents for acetic acid determination, N-glycosidase F, and O-glycosidase were purchased from Boehringer Mannheim, while a chemical deglycosylation kit was purchased from Oxford Glycosystems (Oxford, UK). All chromatography media (Q Sepharose fast flow, Sephacryl S-300 HR, MonoS), and molecular weight markers were obtained from Pharmacia Biotech Inc. (Uppsala, Sweden), whereas ultrafiltration membranes were from Amicon and Millipore. Amorphous and crystalline chitin from shrimp and crab and chitin 50 were a gift from Dr. K. M. Varum (Norwegian Biopolymer Laboratory, Institute of Biotechnology, Trondheim, Norway). All other chemicals were of the highest purity commercially available.

Enzyme Activity Assays—We have employed two different assays for determining chitin deacetylase activity. (i) In a radiometric assay, chitin deacetylase activity was estimated using as substrate partially O-deacetylated chitin (glycol chitin), radiolabeled in N-acetyl groups. The substrate was prepared according to Araki and Ito (6) and was incubated for 30 min at 50 °C (pH 8.5) was used as buffer, and incubation time was 15 min at 50 °C. (ii) In an enzymatic assay, acetate released by the action of chitin deacetylase on various chitinous sub-
strates was determined by the enzymatic method of Bergmeyer (17) via a total volume of 50 ml by ultrafiltration, and subsequently applied onto a Sephacryl S300 HR column. Fractions containing enzyme activity were collected. The protein content was followed by a UV monitor at 280 nm.

Microorganism Cultivation—C. lindemuthianum was grown in shake cultures for 3 days at 25 °C in 2-liter Erlenmeyer flasks containing 500 ml of a medium consisting of 15 g of glucose, 6.6 g of glutamic acid, 1 g of K_2HPO_4, 0.5 g of MgSO_4 \cdot 7H_2O, 1.8 mg of ZnSO_4 \cdot 7H_2O, 1 mg of FeSO_4 \cdot 7H_2O, 0.3 mg of MnSO_4 \cdot H_2O, 0.4 mg of CuSO_4 \cdot 5H_2O, 1 mg of thiamine, and 1 mg of nicotinic acid per 1 liter of deionized water. Medium was inoculated with 2 ml of culture broth (2 liters) was concentrated to a volume of 500 ml by ultrafiltration, and subsequently applied onto a Sephacryl S300 HR column, was concentrated, dialyzed against 20 mM Bis-Tris-HCl, pH 5.8 (buffer A), culture filtrate, and then subsequently loaded onto a Q Sepharose fast flow column (44 mm) previously equilibrated in buffer A. The column was washed with buffer A and the retained proteins were subsequently eluted with a linear gradient of NaCl (30 ml; 0–1 M) at a flow rate of 300 ml/h. Fractions containing enzyme activity were collected. The protein content was followed by a UV monitor at 280 nm.

Enzyme Purification—The enzyme was purified from the culture filtrate of the fungus since preliminary experiments showed that CDA-specific activity was 10-fold higher compared to mycelial extracts. Culture broth (2 liters) was concentrated to a volume of 500 ml by ultrafiltration, dialyzed against 20 mM Bis-Tris-HCl, pH 5.8 (buffer A), and subsequently loaded onto a Q Sepharose fast flow column (44 mm) previously equilibrated in buffer A. The column was washed with buffer A, and the retained proteins were subsequently eluted with a linear gradient of NaCl (2000 ml; 0–0.5 M) at a flow rate of 300 ml/h (Fig. 1).

Some carbohydrate analysis was performed by the Dartmouth Microchemistry facility (Hanover, N.H.). Amino acid analysis was performed after protein hydrolysis in vaporgas phase in constant boiling HCl at 110 °C for 24 h by the method of Spackman et al. (22). Half-cystine was estimated by using a wheat starch ladder polymers of glucose.

Protein Sequencing—The amino-terminal sequence of chitin deacetylase was determined after electroblotting a purified enzyme preparation onto a poly(vinylidene difluoride) membrane. Sequence analysis was performed by the Dartmouth Microchemistry facility (Hanover, N.H.).

Carbohydrate Analysis—Monosaccharide compositional analysis was performed by M-Scan (M-Scan Ltd., Ascot, UK) using gas chromatography/mass spectrometry. For this analysis methanolysis was carried out followed by re-N-acetylation with acetic anhydride and derivatization with trimethylsilyl imidazole (Tris-Sil Z) before injection into the gas chromatography/mass spectrometry instrument.

Protein Determination—Protein content was determined according to the method of Lowry et al. (20) using bovine serum albumin as standard.

Enzyme Deglycosylation—(i) For enzymatic deglycosylation, endoglycosidase F/N-glycosidase F from Flavobacterium meningosepticum (40 milliunits) and O-glycosidase from Diplolococcus pneumoniae were incubated with CDA (10 µg) under denaturing or native conditions overnight at 37 °C in the presence of phenylmethylsulfonyl fluoride (0.5 mM/m). (ii) Chemical deglycosylation was performed by hydrazinolysis according to a modified procedure of Sojar and Bahl (21).
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**TABLE I**

| Step          | Total protein | Total enzyme activity | Specific activity | Yield | Purification |
|---------------|---------------|-----------------------|-------------------|-------|--------------|
|               | mg            | units*                | mg                | %     | -fold        |
| Filtrate      | 635           | 1245                  | 0.002             | 100   | 1            |
| Q Sepharose   | 59.5          | 996                   | 0.017             | 80    | 8.5          |
| 5300          | 9.24          | 714                   | 0.078             | 57    | 39           |
| Mono S        | 0.93          | 203                   | 0.219             | 16    | 110          |

*One unit of enzyme activity is defined as the amount of the enzyme required to produce 1 µmol of acetate/min when incubated with 16.6 nmol of hexa-N-acetylchitohexaose under optimum pH (8.5) and temperature (50°C) conditions.

**TABLE II**

| Amino acid composition of chitin deacetylase | µg | nmol | nmol % |
|---------------------------------------------|----|------|--------|
| Aspartic acid                               | 2.4| 21   | 10.6   |
| Threonine                                   | 1.8| 18   | 9.1    |
| Serine                                      | 1.5| 17   | 8.6    |
| Glutamic acid                               | 1.4| 14   | 7.1    |
| Acid                                        | 1.0| 10   | 5.1    |
| Proline                                     | 1.1| 20   | 10.1   |
| Glycine                                     | 1.6| 22   | 11.1   |
| Alanine                                     | 0.5| 0.5  | 0.3    |
| Cystine                                     | 0.5| 12   | 6.1    |
| Valine                                      | 0.5| 2.5  | 1.3    |
| Methionine                                  | 0.8| 6.8  | 3.5    |
| Isoleucine                                  | 1.6| 14   | 7.1    |
| Leucine                                     | 1.5| 9.1  | 4.6    |
| Tyrosine                                    | 1.4| 9.3  | 4.7    |
| Phenylalanine                               | 1.0| 7.4  | 3.7    |
| Histidine                                   | 1.2| 9.2  | 4.6    |
| Arginine                                    | 0.8| 4.8  | 2.4    |

**FIG. 4.** Electrophoretic pattern of chitin deacetylase. Purified enzyme preparation was electrophoresed on a 10% polyacrylamide gel under denaturing and reducing conditions. Protein bands were visualized by staining with Coomassie Brilliant Blue R. Identities and amount of protein loaded were: Lane 1, culture filtrate (50 µg); lane 2, Q Sepharose eluate (~50 µg); lane 3, Sephacryl 300 eluate (~15 µg); lane 4, MonoS eluate (~5 µg); lane 5, N-deglycosylated enzyme (treatment with N-glycosidase F (~5 µg)).

result in any further reduction in the molecular weight of the enzyme. These results indicate that oligosaccharide chains are N-linked. Carbohydrate and amino acid analysis of the enzyme revealed that the carbohydrate content is 67% by weight of the protein. The carbohydrate part of the protein consists of galactose, mannose, xylose, fucose, N-acetylα-glucosamine, N-acetylgalactosamine, and N-acetylenuraminic acid (Table III). N-Linked profiling analysis of the enzyme showed numerous bands migrating in a range between glucose oligomers, having a degree of polymerization from 4 to 12 (Fig. 5).

Enzyme Activity Characterization—When glycol chitin was used as a substrate, optimum temperature for enzyme activity was determined to be 50 °C, while the optimum pH was estimated to be 8.5. The enzyme was inhibited by Zn²⁺, Mn²⁺, and Cu²⁺ tested as chlorides, even at relatively low concentrations (1 mM), whereas it was not inhibited by Na⁺, K⁺, Li⁺, Mg²⁺, and Ca²⁺ up to 50 mM concentration. A maximum ~3-fold increase in activity was observed with Co²⁺ (0.2 mM concentration), but only when glycol chitosan was used as a substrate. The enzyme was not inhibited by carboxylic acids (acetic acid, formic acid, and propionic acid), even at concentrations up to 100 mM and exhibited almost no loss of activity after preincubation at 50 °C for 45 h (Fig. 5).

Deacetylation of Chitinous Substrates—CDA was active on glycol chitin and carboxymethylchitin (water-soluble chitin derivatives), amorphous chitin (a chemically modified form of crystalline chitin), chitin 50 (40% deacetylated chitin with an average M₉₅ of ~160,000), and N-acetylcysteineformic acid. Incubation
of chitin deacetylase with various N-acetylchitooligosaccharides for 10 min revealed that the enzyme can deacetylate (GlcNAc)$_5$, (GlcNAc)$_6$, and (GlcNAc)$_6$, with approximately the same efficiency (Table IV). Overincubation of CDA with N-acetylchitooligosaccharides also showed that the enzyme requires at least two N-acetyl-$
$-glucosamine residues for catalysis.

DISCUSSION

In this report, we describe the purification of the enzyme chitin deacetylase from the culture filtrate of the fungus C. lindemuthianum to apparent electrophoretic homogeneity. The enzyme is a glycoprotein. From the carbohydrate and amino acid analysis in combination with the apparent molecular mass of the deglycosylated enzyme (~50 kDa), its apparent molecular mass was determined to be ~150 kDa, while its carbohydrate content ~67% by weight. Further incubation of the N-deglycosylated enzyme with O-glycosidase, did not result in any further reduction in the molecular mass of the enzyme, suggesting that oligosaccharide chains are N-linked. The fluorophore-labeled N-glycan migration range was between glucose oligomers, having a degree of polymerization between 4 and 12. Most N-linked oligosaccharides migrate in this region of the gel and usually consist of between 10 and 20 monosaccharide units. The glycosylation pattern of the enzyme is consistent with a mixture of N-linked glycans including the oligomannosidic hybrid and/or complex type.

Although several other purification schemes were also employed in our laboratory resulting in a final enzyme preparation of comparable purity, the one we describe here was chosen for several reasons. It is simple, since it can be completed within 3 days; it employs standard protein purification media and equipment; it is economic, as expensive adsorbents are avoided; and it is easy to scale up. Polyclonal antibodies raised against the M. rouxii CDA do not react with the C. lindemuthianum enzyme, even though we have observed immunological homology of CDAs within the Zygomycetes class (data not shown). Thus, immunoaffinity chromatography based on antibodies against the M. rouxii enzyme, which has been successfully employed for the purification of CDA from M. rouxii to homogeneity in a one-step procedure (12), cannot be effective for the isolation of the enzyme from C. lindemuthianum.

We have used two different assays for the determination of CDA activity. A radiometric assay, using radiolabeled glycol chitin as substrate, proved to be a rapid and sensitive way of screening chromatographic fractions, whereas the estimation of acetate released by an enzymatic method was used for monitoring the deacetylation process of nonradiolabeled substrates (24).

Glycol chitin has been used as a model substrate for the determination of CDA activity (6). Since it is not easy to evaluate, (i) the extent and distribution of derivatization (O-hydroxymethyl groups) in glycol chitin commercially available and (ii) the effect of derivatization on enzyme activity, we have used hexa-N-acetylchitohexaose as a model substrate for the determination of the enzyme activity as described previously (11). When glycol chitin was used as substrate, the optimum temperature for enzyme activity was determined to be 50 °C, similar to all CDAs examined so far, while the optimum pH was
estimated to be 8.5, which is the highest reported. Furthermore, the enzyme is not inhibited by acetate and exhibits a remarkable thermostability.

CDA from C. lindemuthianum appears to exhibit a very narrow specificity, acting only on N-acetyl-α-glucosamine homopolymers, similarly to the enzyme from M. rouxii and A. coerulea, while the corresponding enzyme from A. nidulans was found to exhibit a wider specificity. Overincubation of the enzyme with N-acetylchitooligosaccharides revealed that the enzyme requires at least two N-acetyl-α-glucosamine residues for catalysis. However, shorter incubation times (10 min) indicated that the rate of deacetylation was considerably higher with chitin oligomers having more than three N-acetyl-α-glucosamine residues and that the enzyme can deacetylate \((\text{GlcNAc})_N\) \((\text{GlcNAc})_M\), and \((\text{GlcNAc})_6\) with approximately the same efficiency. In the case of \(M.\) rouxii and \(A.\) coerulea enzymes, the rate of deacetylation was higher the longer the chitooligosaccharide was. Chitin deacetylase from \(A.\) nidulans showed maximum activity with \((\text{GlcNAc})_6\) while it was less active on \((\text{GlcNAc})_5\). In order to test CDA effectiveness in deacetylating chitin and chitosan substrates, two crystalline and two amorphous chitin samples as well as a chitosan substrate (chitin 50) were incubated with the enzyme under the standard assay conditions. When the enzyme was incubated with the 40% deacetylated chitin and various N-acetylchitooligosaccharides for 10 min, the degrees of deacetylation obtained were comparable (Table IV). When crystalline chitin and its chemically modified form, amorphous chitin, were incubated with the enzyme for 24 h, approximately 0.5 and 5% deacetylation was achieved, respectively (Table V). This indicates that the enzyme is not very effective in deacetylating insoluble chitin substrates and that pretreatment of crystalline chitin substrates prior to enzyme addition is necessary, in order to improve the accessibility of the acetyl groups to the enzyme and therefore enhance the yield and rate of the deacetylation reaction. Similar results employing the same substrates and CDA from \(M.\) rouxii have been reported (24). CDA from \(A.\) coerulea was also not active toward crystalline chitin while it was effective in deacetylating colloidal chitin, an amorphous chitin substrate.

The requirements for substrate recognition and the mechanism of enzyme action on N-acetylchitooligosaccharides as well as chitin and chitosan polymers, need to be further studied.

In summary, CDA from \(C.\) lindemuthianum, as compared to all other corresponding enzymes, exhibits different properties, e.g. increased thermostability and different pH optimum, while it is not inhibited by acetate. These properties could potentially be exploited for the effective deacetylation of chitinous substrates.

Even though chitin biosynthesis, enzymology, and cytology in fungi have been extensively studied, there is limited information on chitosan biosynthesis. It has been reasonably suggested that CDA from \(M.\) rouxii is a secreted enzyme and that its function is localized in the periplasmic space (25). Preliminary immunolocalization experiments in this fungus reinforce this suggestion. It has been also recently demonstrated, by immunoelectron microscopy, that CDA from \(A.\) coerulea is localized near the inner face of the cell wall (periplasmic space). In contrast, CDA from \(C.\) lindemuthianum exhibits a 10-fold higher specific activity in the culture filtrate than in mycelial extracts, suggesting that the enzyme may have another (or additional) role than in chitosan biosynthesis in this fungus.

In considering other possible biological roles for CDA the following hypotheses can be envisaged, taking into account the fact that \(C.\) lindemuthianum is a plant pathogen. First, it is known that chitin oligomers (tetramer to hexamer), elicitor lignification in wounded wheat leaves (26), formation of callose and coumarin derivatives in parsley cells (27), as well as in protoplasts and cells of Catharanthus roseus (28). The deacetylated forms of these oligomers do not possess any significant elicitor activity in any of the above systems. On the other hand, it has been reported that chitosan oligomers induce the synthesis of pisatin in pea pods (29) and of proteinase inhibitors in tomato leaves (30). These observations, in combination with our finding that CDA from \(C.\) lindemuthianum is active on chitin oligomers, suggest that the enzyme might play a role in plant pathogen interactions. A possible scenario is that endochitinases of plant and fungal origin may cause the release of chitin oligomers arising from the cell walls of \(C.\) lindemuthianum. These oligomers could act as elicitors for the synthesis of callose, lignin, and phytoalexins, substances which potentially contribute to the plant’s defense mechanism. CDA being extracellular in this fungus could convert these oligomers to their deacetylated forms, thereby diminishing their elicitor activity. Second, plants produce chitinases which are generally thought to contribute to defense against fungal pathogens (31, 32). Deacetylation of cell wall chitin chains in \(C.\) lindemuthianum might make the polymer resistant to degradation by the plant chitinases. However, it has been reported that cell walls of this fungus do not contain chitosan even though they do contain chitin (33). The above hypotheses could be tested once the structural gene has been cloned (work in progress in this laboratory) by carrying out gene disruption experiments in this fungus.

Chitin deacetylases isolated so far have been reported to be involved either in the formation of the cell wall (\(M.\) rouxii, \(A.\) coerulea) in combination with chitin synthetases operating in tandem or in deacetylating chitin oligosaccharides during autolysis after the action of endochitinase on cell walls (\(A.\) nidulans). In this report, a new role for \(C.\) lindemuthianum CDA is proposed, its involvement in plant-pathogen interactions; since the enzyme is extracellular, its cell wall does not contain chitosan, while it is active on chitin oligomers.

The similarities and differences between nodB proteins and CDAs from \(M.\) rouxii and \(C.\) lindemuthianum make these enzymes an attractive system for studying structure and function relationships in substrate recognition and catalysis. Comparative analysis of sequence similarities between these proteins can provide the basis for developing a protein engineering strategy in order to modify the specificity of CDAs and/or nodB protein. The ultimate goal could be the design of an enzyme with improved efficiency for the synthesis of new Nod factors or oligomeric bioactive chitooligosaccharides and polymeric chitosan substrates.

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