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Enzymatic Modification of Chitosan Using Chitin Deacetylase Isolated from *Bacillus cereus*

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Abstract

One of the classes of polysaccharide with a promising economic potential comprises those with biological activities, such as chitosan, which has been optimised over the course of evolution to fulfil many roles in the life of bacteria, fungi and animals. However, there is still considerable leeway for optimisation of these biopolymers to fulfil their many roles in biotechnology. Today, modifications and optimisations are typically done using chemical methods such as acid or alkali treatment. Alternative and/or complementing enzymatic modifications using chitin deacetylase will reduce energy input and environmental impact while at the same time offering the advantage of higher specificity and, thus, potentially yielding novel polymers and/or oligomers with advanced physico-chemical properties and biological functionalities. In the following research work, one of the isolated Chitin Deacetylase (CDA) from *Bacillus cereus* was expressed in *E. coli* pLysS and later purified using Ni-NTA column. The purified product was later characterised for its pH specificity and co-factors.

Keywords: Chitin deacetylase; *E. coli*pLysS; *Bacillus cereus*; Enzyme purification

Introduction

Chitosan is a linear co-polymer of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues linked via ß-1,4-glycosidic bonds. Naturally, chitosan occurs in the cell walls of some fungi, but today's commercial production is by partial chemical de-acetylation of chitin which is extracted from shrimp shell or squid pen wastes. Chitin, the crucial structural component of most fungal cell walls, of the exoskeleton of insects, crabs and shrimps, and of the endoskeleton of squid and of many other animals, is thought to be the second most abundant biopolymer on earth. With an estimated global annual production of 10^{10-11} tons, chitin is an almost inexhaustible renewable resource with its high crystallinity and consequently low solubility in aqueous solvents. However, severely restrict technical and biotechnological applications for chitin, on the other hand, a large number of highly promising applications have been proposed for chitosan, the partially deacetylated counterpart of chitin [1-3]. Deacetylation leads to the formation of a free amino group which conveys positive charge to the polymer at slightly acidic pH values, making it more hydrophilic. As the only polycationic biopolymer, chitosan has a number of intriguing physico-chemical properties in aqueous solution such as spontaneous formation of nanoparticles and an ability to form physical hydrogels. The positive charge is also thought to be at least partly responsible for the broad range of biological functionalities reported, such as antimicrobial activities, disease resistance inducing activities in plants, and wound healing activities in humans.

Today, chitosan is prepared commercially from chitin isolated from shrimp shell wastes of the fishery industries by stepwise chemical de-N-acetylation using hot concentrated alkali. This is the only step for which so far no commercial enzymatic process is available [4]. At the same time, this would be the step to generate chitosan with non-random acetylation patterns by using enzymes with different types of processivity [5,6]. Currently, rather few bacterial and fungal chitin deacetylase enzymes have been characterised in some detail and next to nothing is known about possible processivity.

In the following research the chitin deacetylase from *Bacillus cereus* was expressed in *E. coli* pLysS using pET22b vector and purified using Ni-NTA affinity based column.

Materials and Methods

Cloning bcpda in pet22b

The pET22b vector (250 ng) was double digested with 0.5 µl each of *Bam*HI and *Xho*I (New England Biolabs, NEB, Germany) using NEB buffer 3 supplemented with 0.25 µl BSA to a final volume of 25 µl. The reaction mixture was kept at 37°C for 2 hours followed by heat inactivation at 65°C for 20 minutes to stop the reaction. The reaction mixture was later cleaned using NucleoSpin PCR clean–up Columns (Macherey Nagel, Germany). The CDA was a kind gift by Prof. Bouriotis cloned in pRSET vector. The gene was amplified and *Bam*HI and *Xho*I sites were incorporated in the amplified product. The amplified product was subsequently digested with *Bam*HI and *Xho*I and cleaned using NucleoSpin PCR clean–up Columns (Macherey Nagel, Germany). The CDA insert was later ligated to the pET22b vector using T4 DNA Ligase (NEB, Germany) at 16°C for 16 hours. *E. coli* pLyS competent cells were transformed with the construct and later the size of the construct was confirmed upon digestion with *Bam*HI and *Xho*I on the 1.0% agarose gel.

Expression and purification of CDA gene

Expression in *E. coli* pLysS was induced with 0.1 mm Isopropyl-β-D-Thiogalactopyranoside (IPTG) exposure for 16 h at 18°C. The cells were harvested by centrifugation at 10,000×g for 10 min and then re suspended in lysis buffer (50 mm sodium phosphate, pH-8.0, 2 M NaCl, 40 mm imidazole) to which the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) had been added to a final concentration of 1 mm. The cells were lysed with the sonicator and the lysates were centrifuged at 100,000×g for 15 min. The resultant

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supernatant, containing the His_6-tagged protein, was loaded into a nickel-NTA column (Invitrogen), which was then washed with 10 column volumes of wash buffer (50 mm NaH_2PO_4, 300 mm NaCl, 50 mm imidazole). Nickel-bound His_6-tagged protein was eluted with a two column volume of the elution buffer (50 mm NaH_2PO_4, 300 mm NaCl, 250 mm imidazole) followed by one column volume with 50 mm NaH_2PO_4, 300 mm NaCl, 500 mm imidazole. Fraction size of 0.5 ml was collected and later the purity of proteinwas confirmed by SDS-PAGE and staining with Coomassie brilliant blue R-250. The column bed volume was 2 ml. The resin was bound to the protein at 4°C for 20h and then washed with 20 mm Imidazole and then eluted with two batches of 250 mm and 500 mm Imidazole.

Characterization of the purified CDA

pH optima of BcPdA: The pH range from 3 to 10 was taken with an overlap at pH 6, 7 and 8 to study the effect of the pH and the buffer. The different buffers used were pH 3-6 Na-citrate buffer, pH 6-7 Bis-Tris, pH 7-8 Tris-Cl, pH 8-10 Borate buffer. All the buffers used were of 50 mm. The reaction was carried out at 37°C for 20 h.

Effect of co-factors on the enzymatic reaction catalysed by Bc-pda: All the co-factors used in the study are in the form of their respective chloride salts. The co-factors used in the study were CO^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+} and Ca^{2+}. The enzyme was incubated for 20 h with the substrate and co-factors at 37°C in Bis-Tris buffer 50 mM, pH 7.0.

Effect of concentration of co-factors on the enzymatic reaction catalyzed by Bc-pda: The effect was studied with the inclusion of CO^{2+} in a concentration range varying from 0.5 mM to 10 mM. The reaction products were analysed by TLC.

Effect of temperature on the enzymatic reaction catalysed by Bc-pda: The reaction was incubated at the said temperature and the amount of acetate released was analysed with the Boehringer kit. The reaction was performed in Bis-Tris buffer 50 mM, pH 7.0 with 2 mM CO^{2+}.

Results

The gene for chitin deacetylase was cloned from *Bacillus cereus* into pET22b vector (Figure 1). The resulting construct with six His tag was subsequently expressed in *E. coli* pLysS cells. Thereafter the cells were induced with IPTG at a concentration 1mm and cultivated at 18°C for 20 hours. As it is known that *E. coli* has a leaky expression in all its compartments, different compartments were tested for the expression of the protein. A SDS-PAGE was run and subsequently western blotting was performed (Figure 2). As the extracellular fraction comprised a sizeable amount of the expressed CDA, this fraction was further purified using Ni-NTA matrix with the specifications as per the manufacture (Qiagen, Germany) (Figure 3). The CDA upon purification revealed two bands, the higher band corresponding to around 32 kDa and lower to 30 kDa. The western blotting in figure 2 also bore two bands wherein the higher band may correspond to the protein with the signal peptide and the lower without it which could be a result of partial digestion of the signal peptide by the proteases. After purifying the CDA, the pH optima were determined using different sets of buffers with overlapping buffering range (Figure 4). The pH range of 3-10 was interrogated with an overlap at pH 6, 7 and 8 to study the effect of the pH and the buffer. It is known that co-factors play an important role in the enzyme activity of chitin deacetylase. In lieu of the same, co-factors in the form of their respective chloride salts were included in the assay mixture (Figure 5). It can be noted from figure 5 that the

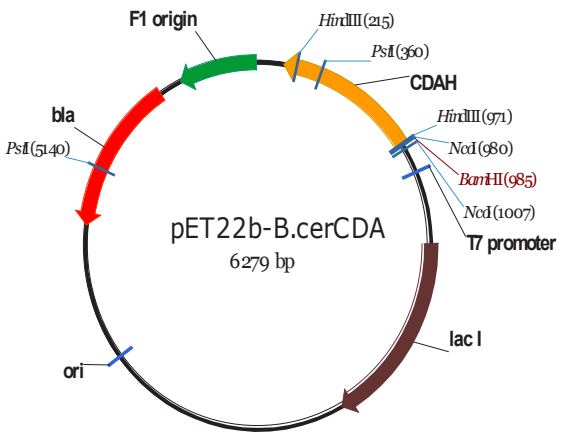

Figure 1: Gene construct of the Peptidoglycan deacetylase in pET-22b.

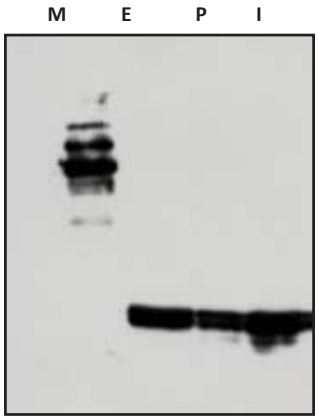

Figure 2: Expression profile of the construct in E. coli pLysS cells. The cells were grown over a period of 5 hours before they reached an OD of 0.5 and were then induced with IPTG of a concentration 1mM and later the cells were grown at 18 C for 20 h before harvesting. Lane 1: Marker (M), Lane 2: Extracellular fraction (E), Lane 3: Periplasmic fraction (P), Lane 4: Intracellular fraction (I).

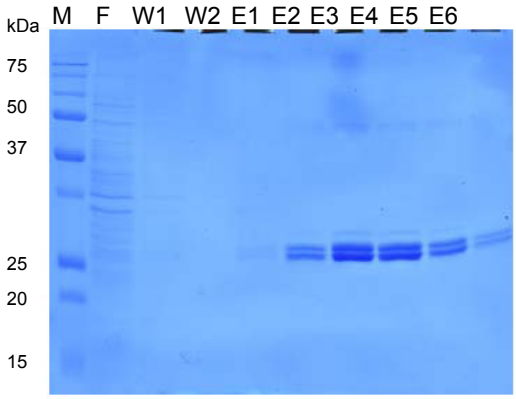

Figure 3: Purification of BcPdA with the Ni-NTA resin using batch mode of purification. The column bed volume was 2 ml. The resin was bound to the protein at 4°C for 20 h and then washed with 20 mM Imidazole and then eluted with two batches of 250 mM and 500 mM imidazole. Lane 1: Marker (M), Lane 2: Extracellular fraction feed (F), Lane 3: Wash1 (W1), Lane 4: Wash 2 (W2), Lane 5: Elution 1 (E1), Lane 6: Elution 2 (E2), Lane 7: Elution 3 (E3), Lane 8: Elution 4 (E4), Lane 9: Elution 5 (E5), Lane 10: Elution 6 (E6).

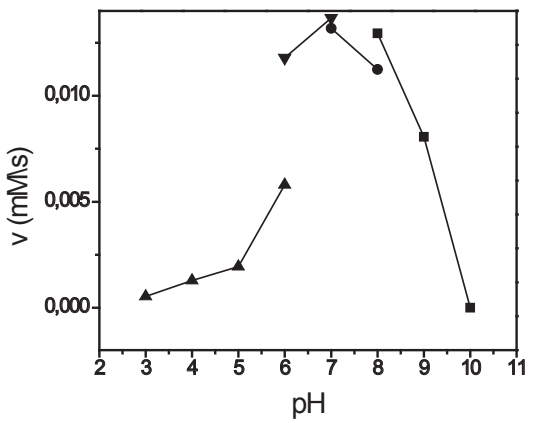

Figure 4: pH optima of BcPdA. The pH range from 3 to 10 was taken with an overlap at pH 6, 7 and 8 to study the effect of the pH and the buffer. The different buffers used were pH 3-6 Na-citrate buffer, pH 6-7 Bis-Tris, pH 7-8 Tris-Cl, pH 8-10 Borate buffer. All the buffers used were of 50 mM. The reaction was carried out at 37°C for 20 h.

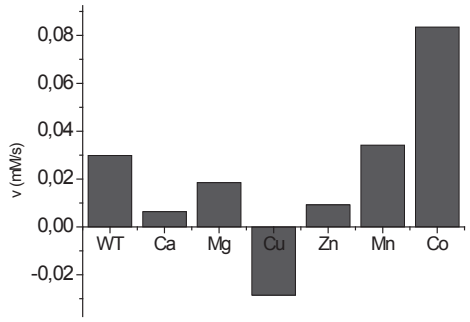

Figure 5: Effect of co-factors on the enzymatic reaction catalyzed by Bc-PdA. All the co-factors used in the study are in the form of their respective chloride salts. The substrate was incubated for 20 h with the substrate at 37°C in Bis-Tris buffer, 50 mM, pH 7.0.

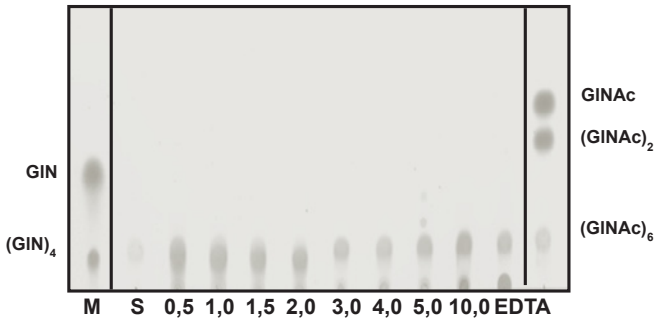

Figure 6: Effect of concentration of co-factors on the enzymatic reaction catalyzed by Bc-PdA. The effect was studied with the inclusion of CO^{2+} in a concentration range varying from 0.5 mM to 10 mM. The reaction products were analyzed by TLC.

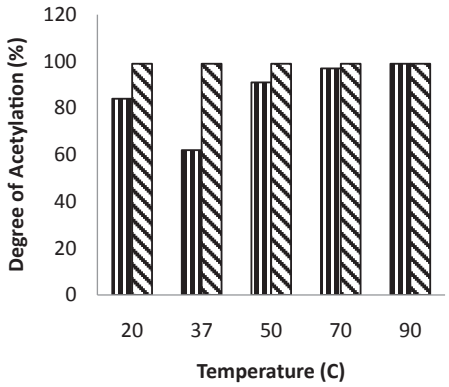

Figure 7: Effect of temperature on the enzymatic reaction catalyzed by Bc-PdA. The reaction was incubated at the said temperature and the amount of acetate released was anylzed with the Boehringer kit. The reaction was performed in Bis-Tris buffer, 50 mM, pH 7.0 with 2 mM CO^{2+}.

enzyme activity was the highest in presence of CO as the co-factor. The concentration of CO was later tested with a range from 0.5 mm to 10 mm (Figure 6). It can be observed that a concentration of 2 mm CO yielded the best activity of the enzyme in terms of its deacetylation activity. At the end the effect of temperature on the activity of CDA was checked with reference to the activity which was checked on the amount of acetate released (Figure 7) with the Boehringer kit and it was found that the enzyme worked well at a temperature of the substrate was incubated for 20 h with the substrate at 37°C in Bis-Tris buffer 50 mM, pH 7.0. Hence the chitin deacetylase was characterised in terms of its pH, temperature optima and the type of co-factor required for its activation.

Discussion

Chitin deacetylase, the enzyme which is instrumental in cleaving the acetyl group from N-acetyl glucasamine units was cloned from *Bacillus cereus* into *E. coli* pLysS cells. pET22b vector was used for expressing the gene into *E. coli* cells. As the gene is toxic to the host cells, the strain with Lysogene was utilized so that a tight control over the expression is maintained. The *E. coli* cells were grown to their exponential phase at the end of which the induction with IPTG at a concentration of 1mm was performed. After induction the cells were grown at 18°C for a period of 16 hours before harvesting the cells. The cells were harvested and different compartments were tested for the expression of the protein. The expressed protein was then purified using the Ni-NTA column as the CDA was tagged with six His at its C-terminus. The purified CDA was observed with two bands corresponding with a higher band of 32 kDa and a lower of 30 kDa. These two bands were also observed in the western blotting signifying the partial digestion of the signal peptide present at the N-terminus. The purified protein was characterised in terms of its pH, temperature optima and the co-factor type and concentration. Glycol chitin was used as the substrate to determine the CDA activity on the gel but since the extent of deacetylation cannot be determined using it as the substrate the latter experiments were conducted with chitohexamers as the substrate which has been well documented earlier [7]. The *E. coli* recombinant enzyme was inactive in the absence of CO^{2+}. In the presence of Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+} not much restoration of the activity was observed while Cu^{2+} imparted a negative effect on the activity (Figure 5). Similar findings were reported with *S. cerevisiae* CDA which displayed activation in the presence of CO^{2+} [8,9]. It has been previously reported that deglycosylation of chitin deacetylases from *C. lindemuthianum* and *S. cerevisiae* resulted in complete loss of enzyme activity, which could be only restored by the addition of CO^{2+} [8]. However, native glycosylated enzymes from *M. rouxii* and *C. lindemuthianum* were active in the absenceof $CoCl_2$. The presence of this metal ion resulted onlyin activation of these enzymes.

Similar works has been cited with fungal CDA wherein Cu and CO have been reported to result in the activation of the enzyme [10]. In another study with peptidoglycan from *Bacillus subtilis* by a group from Japan [11], the enzyme showed more affinity towards Zn^{2+} as compared to CO^{2+} in our study.The pH optima was tested over a range of pH from pH 3-10 with an overlap at pH 6, 7 and 8 to check for the impact of the buffer in addition to the pH. It was at pH 7.0 that the maximum activity was observed. The results were close with another study conducted wherein the bacterial CDA yielded maximum activity at pH 6.0 [12]. Further work on the characterization of the enzyme is underway.

Conclusions

The enzymatic modification of the chitin would result in homogenous deacetylation as against the heterogeneous pattern obtained by the thermochemical modification practiced presently. In addition, the chemical modification of the chitin also results in products with a broad range of molecular weights. As chitosan is presently holding an important industrial importance, the uniformity in terms of the molecular weight and pattern of acetylation is desired. Thus the enzymatic deacetylation of the chitinous material holds a lucrative future with the enzyme from different microbial sources yielding different patterns of deacetylation. Hence a consortium of enzymes can be made use of for different applications of chitosan.

Acknowledgements

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