ISOLATION, MOLECULAR CLONING AND CHARACTERISATION OF TWO GENES CODING CHITIN DEACETYLASE FROM *MUCOR CIRCINELLOIDES* IBT-83

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Abstract

Chitosan is a linear N-deacetylated derivative of chitin, soluble in acetic solutions. The deacetylation of chitin can be achieved enzymatically using chitin deacetylase (ChDa) (EC 3.5.1.41), which hydrolyses the N-acetamido groups of N-acetyl-D-glucosamine residues in chitin and chitosan. Complementary DNA (cDNA), which encodes ChDa, was isolated from M. rouxii s well as other fungi.

Chitin deacetylase activity was detected in partially purified and concentrated crude extract of the protein from Mucor circinelloides IBT-83. Additionally, two open reading frames (ORF), putatively encoding ChDa, were identified and amplified from cDNA of this strain. Each ORF was molecularly cloned and sequenced. Amino acid sequences of ChDaI and ChDaII were predicted, using nucleotide sequences of these cDNA clones, and analysed by means of bioinformatics tools.

Key words: *chitin deacetylase, genes, molecular cloning, sequences analysis.*

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1. Introduction

Chitin, a linear homopolisaccharide composed of (1→4)-2-acetamido-2-deoxy-β-D-glucopyranose units, is one of the most abundant and renewable natural polymer. It is widely distributed in crustaceans such as crab and shrimp, exoskeletons of insects and in cell walls of most fungi. This polymer is insoluble in aqueous solutions and organic solvents. Chitosan is a linear semi-crystaline polysaccharide composed of (1→4)-2-acetamido-2-deoxy-β-D-glucan and (1→4)-2-amino-2-deoxy-β-D-glucopyranose units [1]. This N-deacetylated derivative of chitin is soluble in acidic solutions, and therefore this polysaccharide is more applicable in comparison with chitin. Beneficial properties of chitosan and oligosaccharides are as follows: antifungal, antibacterial [2,3], anti-inflammatory [4], antitumor [5] and neuroprotective [6].

The deacetylation can be achieved enzymatically using chitin deacetylase – ChDa (EC 3.5.1.41), which is associated with mild conditions less burdensome for the environment [7]. ChDa has been identified and partially purified for the first time from extracts of the fungus *Mucor rouxii* [8]. Since then, the activity of this enzyme has been reported to exist in several other fungi [9-11] as well as in marine bacteria and insects [12,13]. Furthermore, fungal ChDa can exist as intracellular (e.g. from *Mucor rouxii* [14], *Rhizopus nigrikans* [15], *Absidia coerulea* [10]) or as extracellular enzymes (e.g. produced by *Colletotrichum lindemuthianum* [11], *Aspergillus nidulans* [9]). The enzymes are acidic glycoproteins with the molecular masses in the range of 25-150 kDa. ChDa often exist in multiple isoforms [9,16]. For nearly all fungal ChDa, the optimum temperature ranges from 50°C to 60°C, while the optimum pH varies from 4.5 to 8.5 [13]. The mechanism of action depends on the source of enzymes and the type of the substrates used. A cDNA encoding ChDa was isolated (from *M. rouxii* [17] and other fungi [18,19]), sequenced and characterised. Furthermore, protein sequence comparisons revealed significant similarities between the enzymes obtained from fungal strains and rhizobial nodulation proteins (NodB), i.e. certain regions of microbial acetylxylanesterases and xylanases, and several uncharacterised open reading frames in *Bacillus* sp, were similar [17,19].

The authors' previous research on enzymatic modifications of chitin and chitosan clearly suggests that *Mucor circinelloides* IBT-83 strain is an efficient source of chitin- and chitosanolytic enzymes. Partially purified intracellular [20,21] and *in situ* immobilised enzymes [22] from this strain (with these specific activities) were characterised and used to produce oligosaccharides in a column reactor [23,24]. The results prompted the authors to make an attempt to isolate the genes encoding enzymes, involved in chitin and chitosan modification. ChDa activity was detected in partially purified and concentrated crude extract of the protein from *Mucor circinelloides* IBT-83. Additionally, two open reading frames (ORF), putatively encoding ChDa, were identified and amplified from cDNA of this strain. Each ORF was molecularly cloned and sequenced. Nucleotide sequences were translated *in silico* and analysed using various bioinformatics tools.

2. Materials and methods

2.1. Materials and strains

Mucor circinelloides IBT-83 strain was obtained from the microbial culture collection available at the Institute of Technical Biochemistry at Lodz University of Technology. *Escherichia coli* TOP10 was used for plasmid construction and molecular cloning. For the purpose of the research, the following items were purchased: Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase, ClonetJET PCR Cloning Kit and Phusion Hot Start II DNA Polymerase – from *ThermoScientific*; PolyATtract® mRNA Isolation System, Wizard® SV Gel and PCR Clean-Up System and PureYield™ Plasmid Miniprep System – from *Promega*; Tri-reagent, PMSF, Luria-Bertani Broth (LB) medium, Chitosan – medium

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molecular weight (MMW, DD 75-85%, viscosity 200 cP) and 1-Bromo-3-chloropropane – from *Sigma Aldrich*; Agar, granulated – from *Difco*.

Mucor circinelloides IBT-83 was grown with shaking at 180 rpm at 30°C for 72 hours in Czapek Dox Broth medium [25] at pH 4.5. Luria-Bertani Agar (LA) medium, supplemented with 100 μg/ml of ampicillin, was used to screen the pJET transformant clones, whereas Luria-Bertani Broth (LB) medium, supplemented with 100 μg/ml of ampicillin, was used to grow the previously selected transformants.

2.2. Fungal extract of chitin deacetylase

The amount of 15 g of fungal mycelium (fresh weight) was mixed with 180 ml of the extracting buffer (10 mM Tris-HCl pH 7.2, supplemented with 1 mM PMSF – final concentration) and homogenised (1 850 rpm) in ice bath for 40 minutes. The homogenate was centrifuged at 12 000 rpm for 30 min. at 4ºC. The obtained supernatant was vacuum-filtered on 0.45 μm filters (*Sartorius*) . The partially purified extract was dialysed (MW cut-off, 4-6 kDa) overnight against 2 liters of 25 mM citric buffer (pH 4.5) and concentrated using *Sartorius vivaflow* 200 system (10 000 MWCO PES). The concentrated crude extract was stored at 4ºC.

2.3. Chitin deacetylase activity assay

Mucor extract was assayed for ChDa activity using the modified method developed by M. M. Jaworska and E. Konieczna [26]. The amount of 5 g of chitosan (MMW, DD 75-85%, viscosity 200 cP) was mixed with approx. 950 ml of 0.1 mM HCl solution (pH 4.0). Next, concentrated HCl was added in small portions (50 – 100 μl) to complete polymer dissolution, which was adjusted with 0.1 mM HCl to the final volume of 1000 ml (pH 4.0). Another step was to use 5 ml of chitosan solution (5 g/L) and 1 ml of *Mucor* extract, preheated separately for 5 min. at 50°C. The reaction was initiated by adding the enzyme into the polymer solution and carried out at 50°C in stirred (300 rpm) sterile falcon tubes. After 240 minutes of incubation, the samples were cooled down and kept at -20ºC. The following step involved the samples being thawed and filtered on 0.2 μm filters, and the released acetic acid concentration was measured. The amount of 900 μl of each filtrates was mixed with 100 μl of propionic acid solution. The resulting concentration of the released acetic acid was measured using the GC-MS method (Stabilwax®-DA, 30 m, 0.18 mm ID, 0.18 μm). One unit of ChDa activity was defined as the amount of enzyme required to produce 0.1 μmol of acetic acid per minute, while being incubated with the chitin solution under standard conditions.

2.4. Isolation of poly(A+)RNA

All solutions were sterile and free from RNase (additive containing 0.1% of DEPC). The mycelium was filtered off, washed with sterile deionised water (RNase-free) and ground to obtain a fine powder in liquid nitrogen. Total RNA was extracted using Tri-reagent (*Sigma Aldrich*). Further purification of poly(A+)RNA was carried out using PolyATtract® mRNA Isolation System (*Promega*).

2.5. Isolation of Chitin deacetylase cDNA genes

Genes potentially encoding ChDa were identified and amplified using specially designed primers and the first strand cDNA as a template for the PCR reaction. The first strand cDNA from *Mucor circinelloides* IBT-83 was prepared using the Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (poly(A+)RNA as a template).

2.6. Transformation

The obtained PCR products were ligated with the pJET1.2/blunt Cloning Vector. The obtained pJET transformants were selected by overnight culturing in LA medium, containing 100 μg/ml of ampicillin at 37°C. Selected transformants were inoculated into liquid LB medium containing 100 μg/ml of ampicillin, and cultured overnight at 37°C. Plasmids DNA was extracted from the selected pJET transformants and sequenced. The resulting sequences were analysed using bioinformatics tools.

3. Results and discussion

This report presents the results of ChDa activity detection in partially purified and concentrated crude extract of the protein from *Mucor circinelloides* IBT-83. Additionally, the report describes the isolation of two cDNA clones potentially encoding this enzyme. Amino acid sequences were predicted using nucleotide sequences of these cDNA clones and compared with the already known protein sequences.

3.1. Prime design

It is well known that cell walls of most fungi contains chitin, while spore walls mostly contain deacetylated derivative, chitosan. Since chitosan formation (followed by enzymatic deacetylation of chitin) has been shown in *M. rouxii* [8], many studies on the structure of genes encoding ChDa have been completed and published. In order to design specific primers, for amplification of genes encoding ChDa, the genome sequence of *Mucor circinelloides* CBS 277.49 (Project ID: 403122, *Joint Genome Institute*) –reconstructed from end-sequenced whole-genome shotgun clones – was aligned, , with the Blast nucleotide database referenced for analysis. It was shown that the genome of *M. circinelloides* putatively contains two genes encoding ChDa. The first match was obtained from multiple alignment of ChDa from *Mucor rouxii* Z19109 with the degree of identity of 88% (1185/1349; gaps = 21/1349; 2%). Another match was obtained with ChDa from *Mucor racemosus* DQ538514, with the identity of 84% (1008/1205; gaps = 31/1205; 3%). Based on these results, it was possible to design specific primers (Table 1).

Table 1. Sequences of the designed primers.

Amplified gene	Primer	Sequence
Gene I ***chdaI***	For_I	5'-ATGCAAATCAAGACATTCGCCC-3'
	Rev_I	5'-TTAAAGTAACAAGGTAGCAATAAAGGCAG-3'
Gene II ***chdaII***	For_II	5'-ATGTATATCAAAATCTCTGCTATCGCAG-3'
	Rev_II	5'-TTAGAGAAAATGTAAGCAGCAACGGC-3'

3.2. Chitin deacetylase activity evaluation

Taking into account the reports to be found in literature, there are many examples of intracellular and extracellular ChDa; The activity of this enzyme was estimated both in the partially purified *M. circinelloides* IBT-83 mycelial extract and in the culture filtrate. The ChDa activity was found only in the partially purified and concentrated crude extract. The enzyme stored at 4ºC was stable for 2 weeks. After this period, the activity decreased significantly (Table 2.).

Table 2. The acetate (AcOH) production values obtained after chitin deacetylation and ChDa activity estimated for the partially purified mycelial extract and culture filtrate of *M. circinelloides* IBT-83.

Sample	Released AcOH [mg/l]	ChDa activity [mU/ml]
Fresh – after extraction		
Fresh cell-free extract	53.3	23
Culture filtrate	0	0
Cell-free extract after storage at 4ºC for:		
1st week	62.4	25.9
2nd week	58.3	24.3
3rd week	38.1	15.9
4th week	37.3	15.5

3.3. Detection of chitin deacetylase cDNA genes and analysis of cDNA clone sequences

Genes potentially encoding ChDa were identified and amplified using specially designed primers. The first strand cDNA of *Mucor circinelloides* IBT-83 was used as a template for the PCR reaction. The attached electrophoresis gel (Fig. 1) shows that two products of different length – approx. 1250-1300 (*chdaI*) and 1350-1400 bp (*chdaII*) – were detected in this PCR reaction.

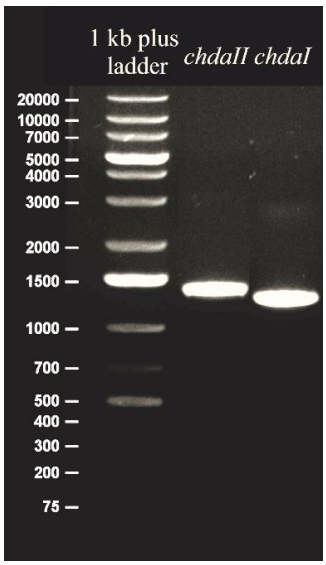

Figure 1. Agarose gel electrophoresis of PCR products. The first strand cDNA was used as a template (35 V; 1 % (w/v); 3.5 h).

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For further characterisation of the genes potentially encoding ChDaI and ChDaII, the obtained oligonucleotides were ligated with the pJET1.2/blunt vector and used to transform *E. coli* TOP 10 cells. Plasmid DNA, extracted from the selected pJET transformants, were sequenced and characterised.

The obtained sequences revealed two open reading frames (ORF). The first ORF of 1269 nucleotides potentially encoded a 423-amino-acid protein labelled as ChDaI, and the other ORF of 1365 nucleotides potentially encoded a 455-amino-acid protein, labelled as ChDaII. The obtained amino acid sequences were predicted by *in silico* translation of nucleic acid sequences into their corresponding peptide sequences. A search through available protein databases, using the deduced amino acid sequences of ChDaI and ChDaII, revealed striking similarities of the sequences with the rhizobial nodulation proteins (NodB). This conserved region was assigned as the polysaccharide deacetylase domain or the NodB homology domain, discovered in many proteins as in the following examples: *Amylomyces rouxii* ChDa (UniProtKB - P50325), *Streptococcus pneumoniae* peptidoglycan deacetylase (PDB entry 2c1g), *Bacillus subtilis* polysaccharide deacetylase (PDB entry 1ny1), *Encephalitozoon cunculi* ChDa (PDB entry 2vyo), *Helicobacter pylori* peptidoglycan deacetylase (PDB entry 4ly4). Further analysis of conserved polysaccharide deacetylase domain revealed (for both proteins) that a 193-residue functional domain (from *Amylomyces rouxii* ChDa UniProtKB - P50325) was located in the middle part of the amino acid sequence (Fig. 3).

Eleven out of 27 asparagine residues in the deduced amino-acid sequence of ChDaI and eight out of 26 asparagine residues in ChDaII are part of the tripeptide sequence Asn-X-Ser/Thr. This sequence is referred to as N-linked glycosylation sequon, and thus it serves as potential N-glycosylation sites, among which three of them (Asn183, Asn322 and Asn339) coincide in both amino-acid sequences, as predicted within the NodB homology domain. The deduced ChDaI amino-acid sequence has additional N-glycosylation site (Asn338) within the conserved polysaccharide deacetylase domain (Figure 2). The remaining N-glycosylation sites, which are located outside of the homology domain in most cases, are different for ChDaI and ChDaII sequences, except Asn54, Asn367 and Asn383. These differences may indicate that filamentous fungi *Mucor circinelloides* IBT-83 produced two ChDa of different properties.

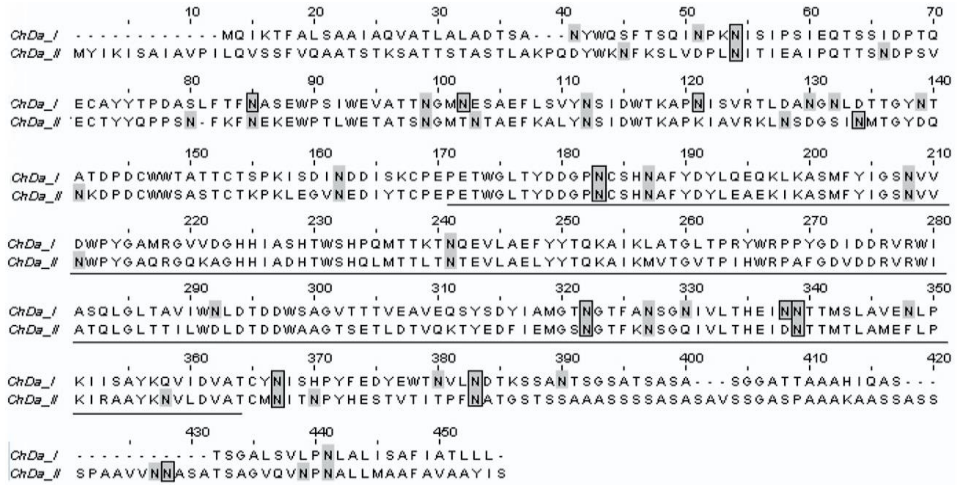

Figure 2. Deduced primary structure of ChDaI and ChDaII from *Mucor circinelloides* IBT-83. Predicted N-glycosylation sites are in bold and boxed. The conserved polysaccharide deacetylase domain is underlined.

The molecular masses and pI values estimated for the predicted ChDaI and ChDaII (non N-glycosylated) amino acid sequences are as follows: pI: 4.44; MW: ≈ 46.1 kDa and pI: 5.01; MW: ≈ 49.3 kDa, respectively. Most of the previously reported ChDa are glycoproteins with the molecular mass in the range of 25-80 kDa, although 150 kDa molecular mass was found in *C. lindemuthianum* DMS 63144. Moreover, carbohydrate content of the enzyme from different sources is very diverse as it ranges from 20 to 70% [13]. Based on the reports to be found in literature, it can be observed that most of the intracellular ChDa have a higher molecular mass than extracellular forms. Taking into the consideration the fact that ChDa activity was indicated only in the partially purified and concentrated crude enzyme extract in the research, the authors can suggest that at least one of the analysed amino acid sequences is an intracellular ChDa.

Multiple sequence alignment of the sequences analysed in the course of the research (with a sequence of crystallised and well-known ChDa from *Colletotrihum lindemuthianum Cl*CDA (PDB 2lWO) and the remaining representatives of the ChDa family) shows that the predicted ChDaI and ChDaII share relatively high identity with deacetylases from the majority of others zygomycetes, including ChDa from *Rhizopus delemar* EIE76781 (77% and 64%), *Rhizopus microsporum* CEJ01745 (75% and 64%), *Rhizopus oryzae* AEC33271 (62% and 72%), two ChDa from *Mucor racemosus* ABG22619 (62% and 80%) and ABG73111 (66% and 59%). The obtained results clearly demonstrate that ChDaI and ChDaII contain five well-conserved catalytic motifs, which make up the active site of the deacetylase domain in the CE-4 family (Fig. 3) [11].

The published experiments on xylan esterases [27], ChDa [28] and peptidoglycan deacetylase [29] conclude that activity of these CE-4 esterases depends on the presence of a divalent cation, preferably cobalt or zinc. Fig. 3 shows that proteins predicted sequences have a conserved Metal-Binding Triad [11,27], consisting of two histidines from MT2 and an aspartic acid from MT1. This His-His-Asp metal-binding triad is conserved throughout the CE-4 family, with the exception of *Bs*PdaA and the recently characterised xylan esterase *Ct*CE4 [28], which have only two metal coordinating residues. The results obtained from the multiple sequence alignment indicate that ChDaI and ChDaII also contain residues involved in binding the acetate group from chitin and/or chitosan. It is worth mentioning that Blair et al. [11] in his research shows one of the acetate oxygens interacting with Asp182 (MT1), which in turn is tethered by a buried Arg275 (MT3). The same oxygen is also bonded with His403 (MT5) that interacts with Asp305 (MT4) (Fig. 3). The backbone nitrogen from Tyr145 (MT3) forms a hydrogen bond with the other acetate oxygen.

Based on the diverse locations of ChDa in different fungi, these enzymes have been divided into two subgroups as follows: intracellular ones, which are secreted into the periplasm (*Mucor rouxii* [14] and *Absidia coerulea* [10]); and extracellular ChDa, which are secreted into the culture media (*Colletotrihum lindemuthianum* [11] and *Aspergillus nidulans* [9]). It is mostly the small signal peptide, presented at the N-terminus of the majority of proteins, which is responsible for directing the newly synthesised proteins into the different locations (secretion, membrane proteins). According to the presented results, ChDaI and ChDaII share relatively high identity with deacetylases from the majority of other zygomycetes, which generally are periplasmic enzymes. The predicted ChDaI and ChDaII amino acid sequences were checked in order to detect signal peptides, which potentially are responsible for directing proteins into the respective areas of the cell or the extracellular medium. The obtained results showed that both putative ChDa amino acid sequences contain the signal peptides. N-terminus of the ChDaI shares 100% identity with the signal peptide form *Amylomyces rouxii* (*Mucor rouxii*; UniProtKB - P50325) with the cleavage site

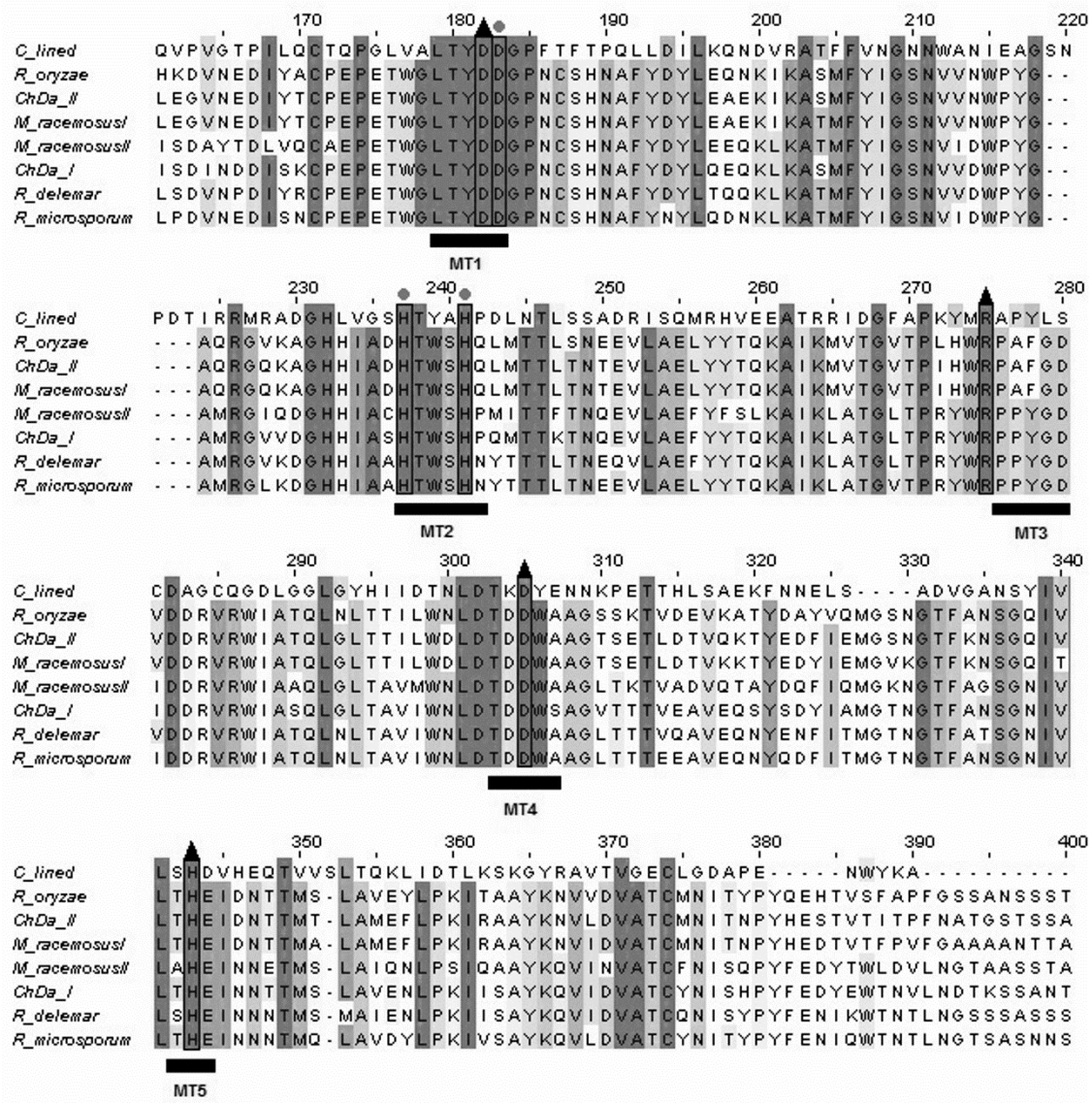

Figure 3. Sequence alignment of CE-4 family members. The sequences of ChDaI, ChDaII and six chitin deacetylases (with high identity) are shown: *Rhizopus delemar* EIE76781, *Rhizopus microsporum* CEJ01745, *Rhizopus oryzae* AEC33271, *Colletotrihum lindemuthianum* AAT68493, *Mucor racemosus* I: ABG22619 and II: ABG73111. The five CE-4 motifs (MT1-MT5; highlighted seq.) are responsible for metal binding (frames marked with circles), the catalytic residues (frames marked with triangles). Shaded areas: identical amino acids – dark grey, conservative amino acid substitutions – bright grey.

between position 21 and 22. ChDaII also shares complementarity with the signal peptide from *Amylomyces rouxii* (identity 35 %) with the cleavage site after 33 aa.

4. Conclusions

The obtained results and bioinformatic analysis of nucleotide sequences, used as the basis for predicting amino acid sequences of ChDaI and ChDaII, show that *Mucor circinelloides* IBT-83 produces two proteins, which putatively exhibit the ChDa activity. Detection of the presence of ChDa activity only in the concentrated crude extract and the occurrence of signal peptides in both amino acid sequences clearly indicate that ChDa from *Mucor circinelloides* IBT-83 represent intracellular enzymes. The deduced amino acid sequences demonstrate that ChDaI and ChDaII contain five well-conserved catalytic motifs, which comprise the active site of the deacetylase domain in the CE-4 family. Additionally, the predicted sequences include a conserved His-His-Asp metal-binding triad, which is conserved throughout the CE-4 family as well.

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