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Kinetic evidence of catalytic residues in the activity centre of chitin deacetylase from *Absidia coerulea* vel *orchidis*

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Abstract
The deacetylation process of chitin or chitosan is carried out on industrial scale by chemical reaction with concentrated NaOH or KOH solution, but an enzymatic process is also possible. Enzymatic deacetylation with chitin deacetylase (EC 3.5.1.41) is non-destructive for polymer chains, and that is why recently it has been investigated more intensively. The structure of the enzyme is important information as it helps to better understand the enzyme action. Chitin deacetylase's primary and secondary structures were presented in literature and were the basis for the mathematical modelling of the 3D tertiary structure. However, the mathematical model for the activity centre has never been confirmed experimentally.
This paper presents the experimental confirmation of a computer modelling of the catalytic residues in the activity centre of extracellular chitin deacetylase from *Absidia coerulea* vel *orchidis*. Based on kinetic studies, amino acids responsible for enzyme activity were determined experimentally as aspartic acid and glutamic acid or as two aspartic acid residues.

Keywords
chitin deacetylase, activity centre, kinetic studies

1. INTRODUCTION

The deacetylation of chitin is an important process that converts chemically resistant chitin into chitosan, a polymer with high biological activity. This conversion turns non-reactive N-acetylglucosamine (GlcNAc) units of chitin into reactive glucosamine (GlcN) units. This higher reactivity plays an important role in such applications of chitosan as wound healing agent (Wang et al., 2024; Zhang et al., 2024), food preservative (Shahbaz et al., 2023), enzyme immobilization (polymer can be used as a carrier) (Bai et al., 2023) or wastewater treating material (Abdullah et al., 2024). The deacetylation process is carried out on industrial scale by chemical reaction with concentrated NaOH or KOH solution (45–50%), but enzymatic modifications of the surface of chitin particles are also possible (Jaworska and Roberts, 2016; Jaworska et al., 2024). Enzymatic deacetylation with chitin deacetylase (CDA) is non-destructive for polymer chains, and that is why it has recently been investigated more intensively. Chitin deacetylase (EC 3.5.1.41) is the only known enzyme able to hydrolyze acetamido bond in GlcNAc units of chitosan or chitin and convert them into GlcN units, releasing acetic acid:

$$-\mathrm{GlcNHAc} + \mathrm{H_2O} \xrightarrow{\text{chitin deacetylase}} -\mathrm{GlcNH_2} + \mathrm{AcOH}$$

Scheme 1. Enzymatic deacetylation of N-acetyl glucosamine unit of chitin or chitosan.

This enzyme was found in bacteria, fungi, yeast and insects (Zhao et al., 2010). Among them, chitin deacetylase from fungi was investigated the most intensively due to easy separation and possible industrial application. Fungal chitin deacetylase exists in two forms – as an intracellular enzyme for example in *Rhizopus stolonife* (El Ghaouth et al., 1992) and *Colletotrichum gloeosporioides* (Pacheco et al., 2013), or as an extracellular enzyme, secreted by such microorganism as *Colletotrichum lindemuthianum* (Kang et al., 2014). However, some fungi can produce the enzyme in both forms, depending on the phase of growth and growth conditions (e.g. *Absidia coerulea* (Win and Stevens, 2001), *Absidia glauca, Mucor rouxi* (Win et al., 2000)). The primary structure of chitin deacetylase was reported for the first time by Kafetzopoulos et al. (1993) for the enzyme separated from *Mucor rouxi*. After that, Tsigos and Bouriotis (1995) presented the amino acid composition of chitin deacetylase from *Colletotrichum lindemutianum*. Both enzymes were purified to homogeneity, and the amino acid sequence was analyzed. Additionally, Kafetzopoulos et al. (1993), based on the amino acid sequence, identified the primary DNA structure coding the chitin deacetylase. Later, the primary structure of the enzyme, predicted based on DNA structure, was published for several other chitin deacetylases separated from:

- bacteria (Liang et al, 2023); *Bacillus subtilis* (Maw et al., 2002); *Bacillus thuringiensis* (Ali et al., 2013), *Vibrio cholerae* (Andrés et al., 2014); *Vibrio parahaemolyticus* (Hirano et at., 2015),
- yeast (*Saccharomyces cerevisiae* (Maw et al., 2002); *Cryptococcus laurenti* (Sarkar et al., 2017)),
- fungi (Liang et al, 2023); *Gongronalla butleri* (Maw et al., 2002); *Colletotrichum lindemutianum* (Blair et al 2006); *Rhizopus nigricans* (Jeraj et al, 2006); *Mucor circinelloides* (Kaczmarek et al. 2016); *Aspergillus nidulans* (Liu et al., 2017); *Aspergillus niger* (Bonin et al., 2021), and
- insects (Liang et al., 2023); *Antonospora* (Maw et al., 2002).

Knowing the primary structure, the secondary structure of chitin deacetylase was assumed for several source organisms. The tertiary structure was presented for the first time by Blair et al. (2006) for the enzyme secreted by *Colletotrichum lindemutianum*. From that moment, the ribbon 3D structure of chitin deacetylase was also presented for *Entamoeba invadens* (Das et al., 2006), *Bacillus thuringiensis* (Ali et al., 2013), *Aspergillus nidulans* (Liu et al., 2017), *Aspergillus niger* (Bonin et al., 2021), *Cryptococcus laurenti* (Sarkar et al., 2017).

Comparing the amino acid sequence established for several chitin deacetylases, five main conserved regions and motifs responsible for connection with substrate and cofactor binding were evaluated (Blair et al., 2005). Motif 1, described by Blair et al. (2006) for *Colletotrichum lindemuthianum*, is based on two aspartic acid residues responsible for binding zinc or cobalt (as cofactors) and acetic acid (product of the reaction). Motif 2 consists of histidine, serine and threonine residues, where the histidine residues are responsible for metal binding. Motif 3 is supposed to coordinate functions of other motifs and is responsible for active groove, together with tryptophan from motif 4. Leucine and histidine from motif 5 are responsible for product binding (Blair et al., 2006). However, Dixit et al. (2008) also presented the differences between those regions for several CDAs, e.g.: separated from *Tribolium castaneum, Drosophila melanogaster, Anopheles gambiae, Apis mellifera, Clostridium thermocellum, Streptococcus pneumoniae, Streptococcus lividans, Rhizobium meliloti, Bacillus subtilis* and *Colletotrichum lindemuthianum*. The differences were more significant when one compared the enzymes coming from different Kingdoms, but even for the fungi, they were noticeable.

The structure of chitin deacetylase is important information, as it helps better understand the mechanism of the enzyme action. The ribbon 3D structure of several CDA published in the literature (see above) was established based on X-ray crystallographic analysis followed by mathematical modelling. Mathematical modelling also suggested the configuration of the activity centre of the enzyme. All of those studies were based only on mathematical and computer modelling, which means that the structure of the enzyme presented up to now was not confirmed by any additional experiments. It must be strongly emphasized that the amino acids responsible for the reactivity of chitin deacetylase (linkage with a substrate) have not been confirmed experimentally. The present paper wants to fill this gap.

The structure of the activity centre can be evaluated based on pH influence on kinetics parameters in Michaelis–Menten equations (V_{max}, K_M) according to the method proposed by Dixon and Webb (1979) and also described by Bisswanger (2002) and Segel (1993). In this method, the kinetics parameters should be transformed into V_{max}/K_M vs pH plot. The plot has a bell-like shape. The line plotted in the half of the height of the maximum value gives values of pKa's correlated with amino acids responsible for the enzyme activity. Taylor proposed a similar method (Taylor, 2004). The data were plotted as $-\log(k_{3,0}/k_{3,exp})$ vs pH (where $k_{3,0}$ is a reaction rate constant at optimal pH, $k_{3,exp}$ is a reaction rate constant for experimental pH, other than optimal value). The plot approaches an asymptote with a slope equal to 1.0 for a low pH value and another one with a slope equal to –1.0 for a high pH value, with a horizontal segment in between. The asymptotes intersect with the plot at two points for which the pH corresponds to the pKa values correlated with amino acids responsible for the enzyme activity. Once the pKa values are known, the amino acids involved in the catalytical activity of the enzyme can be recognized, as each amino acid has its own typical pKa value.

It was shown earlier that the enzymatic deacetylation of N-acetylglucosamine units of chitin or chitosan with chitin deacetylase follows the model proposed by Michaelis and Menten regardless of the source of the enzyme (Alfonso et al., 1995; Amorim et al., 2005; Aspras et al., 2017; Hekmat et al., 2003; Jaworska, 2012; Martinou et al., 1998; Tokuyasu et al., 1996), so the presented method can be applied to any chitin deacetylase to experimentally confirm amino acids responsible for the enzyme activity.

Based on kinetic investigations, the studies aimed to present the experimental confirmation of the catalytic residues in the structure of the activity centre of chitin deacetylase from *Absidia coerulea*. The amino acid residues identified based on the experimental, kinetical analysis were then compared with amino acid residues presented for CDAs in the literature.

2. MATERIALS AND METHODS

2.1. Chitin deacetylase

Chitin deacetylase (EC 3.5.1.41) is not commercially available and it was partially purified from the culture medium of *Absidia coerulea* NCAIM F 00642 (late logarithmic phase of growth) in prior experiments. Fungi were cultivated on YPG nutrient medium at 26 °C, pH 5.5 in a batch culture, according to Jaworska and Konieczna (2001). The culture broth was separated from biomass by centrifugation (3300 *g*, 10 minutes) and filtration at membrane filters (0.45 μm, cellulose nitrate). Further, the solution was purified by ultrafiltration using membrane module Vivaflow 50 (Sartorius, cut off 30 kDa), followed by diafiltration (the same module), in which HCl (pH 4.0) was used. The enzyme solution was stored for up to 5 days at +4 °C. The concentration of the proteins was equal to $C_{prot} = 50.17$ μg/mL and total amount was assumed as chitin deacetylase ($C_{enz} = 0.717$ nmol/mL, molecular weight of the enzyme 70 kDa).

2.2. Chitosan

Chitosan from shrimps (BioLog Heppe, Germany) with the degree of acetylation (DA) of 23% and medium molecular weight (viscosity of 1% solution in 1% acetic acid was 200 mPa),

according to the information of the producer, was used in all experiments. Chitosan (4.0 g) was mixed with 800 mL of pure water, and HCl solution (1%) was added dropwise under pH-controlled conditions (pH in the range from 3.0 to 5.5). The solution was filtered on paper filters with a cut-off of 1 µm to remove insoluble residues. The solution was enhanced to 1000 mL with HCl (the same pH value). The concentration of the prepared chitosan solution was confirmed by the gravimetric method. *The lower concentrations (in the range from 1800 nmol GlcNAc/mL to 4900 nmol GlcNAc/mL) were obtained by mixing in volumetric flasks the concentrated chitosan solution with the HCl solution having the same pH.*

The concentration of N-acetylglucosamine units (the actual substrate) was calculated based on the mass of chitosan and its acetylation degree.

2.3. Reaction rate of enzymatic deacetylation of chitosan

The reaction rate of enzymatic deacetylation of chitosan was determined using the initial rate method. 25 mL of chitosan solution of proper concentration was added to a 50 mL reactor and incubated at 45 °C for 15 minutes, with continuous stirring (200 rpm). 2 mL of chitin deacetylase solution was added to start the reaction (all experiments were carried out with the enzyme from the same batch to avoid changes in enzyme activity). In appropriate time intervals, 2 mL samples were collected and mixed immediately with 0.1 mL of 1 M NaOH to stop the reaction. Samples were centrifuged (10 minutes, 3300 *g*) to separate the precipitated chitosan. The supernatant was collected, and the concentration of acetic acid was determined chromatographically.

2.4. Analytical methods

The concentration of acetic acid was analyzed using the HPLC method described by Jaworska (2012). The HPLC system consisted of a HyperREZ XP Organic acid column and HyperREZ XO Carbohydrate H^+ Guard Column (at 60 °C). Isocratic system Varian ProStar 210 was used with a refractometer detector Varian ProStar 350 and water (0.5 mL/min) as an eluent.

Protein concentration was measured using Bradford method with Coomassie Brilliant Blue (Bio-Rad, USA). The calibration curve was prepared using bovine serum albumin as a standard.

3. RESULTS

Kinetics parameters (K_M, k_3) in Michaelis–Menten eq. (1)

$$\vartheta = \frac{k_3 \cdot C_{enz} \cdot C_{GlcNAc}}{K_M + C_{GlcNAc}} \quad (1)$$

are usually evaluated in optimal conditions, and the influence of pH on them is rarely investigated. In the optimal pH, the tertiary structure of an enzyme is optimal, so its activity is the highest, which is important for the industrial application of enzymes. While changing pH conditions from optimal to more acidic or alkaline, the tertiary structure changes due to environmental interaction. Additionally, the protonation of amino acids responsible for cleavage of the substrate can also change, thus, k_3 and K_M values change as both parameters depend on pH (Copeland, 2000; Segel, 1993). However, by investigating the influence of pH on kinetic parameters, one can also assess the conformation of the activity centre of the enzyme. It is a good tool for confirming the mathematical modelling of the enzyme tertiary structure and the structure of the activity centre through experiments.

The enzymatic deacetylation of chitosan can be described by the Michaelis–Menten equation, and thus, the Dixon and Webb or Taylor's methods can be used to evaluate the amino acids in the activity centre of the enzyme. The present experiments were carried out in the range of pH 3.0–5.5 as chitosan is insoluble in media with pH above 5.5 (gely/solid particles of chitosan change the conditions of experiments from conditions where substrate is soluble to conditions where substrate is insoluble, so evaluated parameters cannot be compared). The reaction rate was measured using the initial rate method. The possibility of application of the Michaelis–Menten equation was confirmed with a Lineaver-Burk plot (concentration of the substrate corresponded to GlcNAc units concentration), Fig. 1, and then parameters in Michaelis–Menten equation were evaluated based on experimental data and using non-linear-approximation, Table 1.

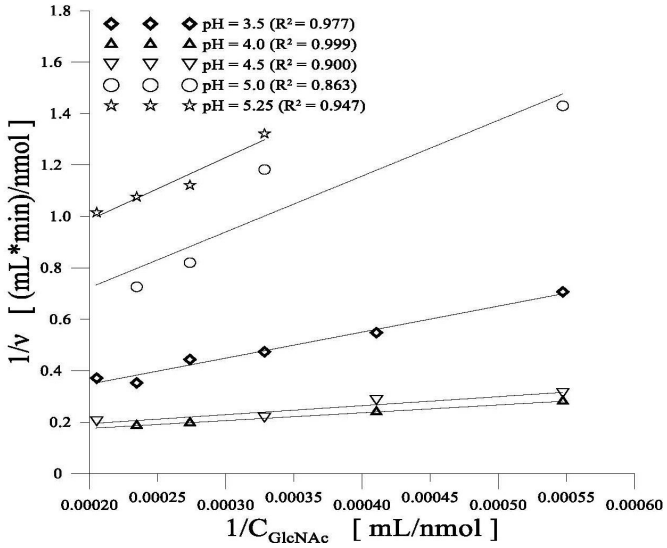

Figure 1. The Lineweaver–Burke plot for enzymatic deacetylation of chitosan; C_{enz} = 0.057 nmol/mL.

The presented data confirmed the optimal pH value as 4.0, as the highest activity of the enzyme was observed for that pH value. It was interesting to notice that the activity of chitin deacetylase at pH 3.0 and 5.5 was very low. For pH 3.5, it was

Table 1. The kinetic parameters of the Michaelis–Menten equation for different pH values.

pH	K_M [nmol/mL]	V_{MAX} [nmol/mL/mi	k_3 [1/min]	R^2
3.0	Activity below the detection limit			
3.5	7193 ± 6	6.87 ± 0.62	129.4	0.974
4.0	2643 ± 2	8.70 ± 0.70	163.7	0.998
4.5	27950 ± 3	8.05 ± 0.57	151.4	0.879
5.0	6977 ± 5	3.46 ± 0.32	65.1	0.879
5.25	9762 ± 8	2.00 ± 0.34	37.6	0.944
5.50	Activity below the detection limit			

probably connected with fast enzyme inactivation due to conformational changes, while for pH 5.5, it might be connected with partial precipitation of chitosan. For chitin solid particles, GlcNAc units available for enzymatic deacetylation are present only at the surface of a particle, so their number is much smaller compared to units available in the soluble polymer. This is probably the reason for the low reaction rates observed.

Based on evaluated parameters, the (V_{MAX}/K_M) vs pH plot (Fig. 2A) as well as ($-\log(k_{3,0}/k_{3,exp})$) vs pH plot (Fig. 2B) have been drawn:

The pK_a value is characteristic for an amino acid, so knowing it, we can determine the amino acids playing the main role in the activity centre. Based on the influence of pH on parameters in the Michaelis–Menten equation (K_M and k_3), the pK_as of amino acids involved in the catalytic activity of the enzyme were determined, as shown in Table 2.

Table 2. The pKa values determined based on experimental data.

	pKa_1	pKa_2
Dixon and Webb's method	3.65	4.76
Taylor's method	–	4.47

The pKa values for amino acids of chitin deacetylase from *A. coerulea* evaluated in both methods are in agreement. Those pKa values are characteristic for aspartic acid (pKa: 3.0–4.7) and glutamic acid (pKa: 4.4) (Marangoni, 2003). Therefore, the catalytic residues in the activity centre are probably aspartic acid and glutamic acid, but it is also possible that there are two residues of aspartic acid in the activity centre.

4. DISCUSSION

The extracellular chitin deacetylase from *Absidia coerulea* was used for investigations focused on the influence of pH on the enzymatic deacetylation of chitosan. Based on the kinetics

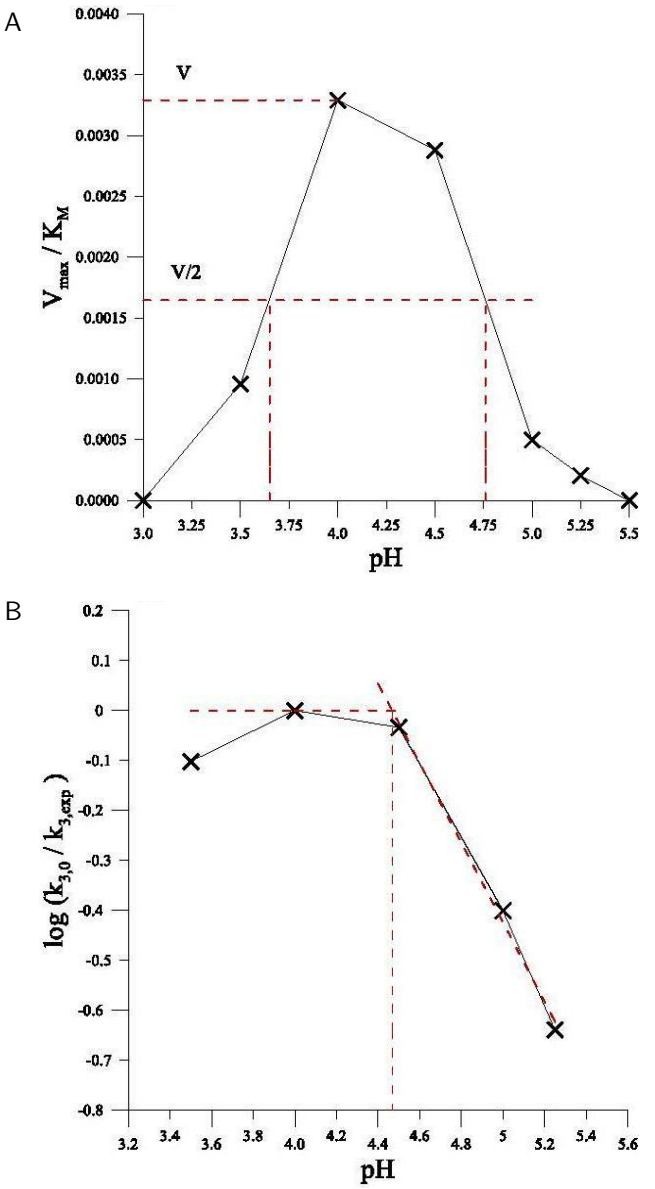

Figure 2. Evaluation of pKa values of amino acids present in the activity center according to Dixon and Webb method (A) and Taylor method (B).

studies, the pKa values of amino acids responsible for the activity of chitin deacetylase were evaluated as $pK_{a1} = 3.65$ and $pK_{a2} = 4.76$ according to Dixon and Webb's method and as $pK_{a2} = 4.47$ according to Taylor's method. These values were characteristic for glutamic acid and aspartic acid. However, since the pKa value of aspartic acid is in the range of 3.0–4.7 and the evaluated value of pKa is 4.76 (or 4.47), it is possible that the second amino acid in the activity centre is also aspartic acid instead of glutamic acid. We compared our results with data published for chitin deacetylase separated from other origins, and we have found that our experimental data confirm mathematical modelling results presented by Blair et al. (Blair et al., 2005; Blair et al., 2006) for chitin deacetylase from *Colletotrichum lindemuthianum*, and by Dixit et al. (2008) for chitin deacetylase from *Tribolium castaneum*,

Drosophila melanogaster, Anopheles gambiae, Apis mellifera, Clostridium thermocellum, Streptococcus pneumoniae, Streptococcus lividans, Rhizobium meliloti, Bacillus subtilis and also *Colletotrichum lindemuthianum*. They suggested that both aspartic acid and glutamic acid should be observed in the structure of five main motifs responsible for enzyme activity. For most of the CDAs, two aspartic acid residues are present in motif 1 and are thought to be responsible for binding cofactors (zinc and cobalt) or for binding products (acetic acid). Motif 4, responsible for the active groove, contains at least one aspartic acid residue in each type of CDA. In motif 5, the aspartic acid residue is probably responsible for product binding. Therefore, for the first time, the results of mathematical and computer modelling data obtained by (Blair et al. 2005; 2006) and Dixit et al. (2008) have been confirmed experimentally.

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SYMBOLS

C_{enz}	concentration of enzyme, nmol/mL
C_{GlcNAc}	concentration of N-acetylglucosamine units, nmol/mL
CDA	chitin deacetylase
DA	acetylation degree, %
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
k_3	reaction rate constant, 1/min
$k_{3,0}$	reaction rate constant at optimal pH conditions, 1/min
$k_{3,exp}$	reaction rate constant at pH other than optimal, 1/min
K_M	Michaelis constant, nmol/(mL·min)
pKa	acid dissociation constant
V	reaction rate, nmol /(mL·min)
V_{max}	maximum reaction rate, nmol /(mL·min)
YPG	Yeast extract, Peptone, Glucose

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