THE ENERGY OF ACTIVATION OF INTRACELLULAR AND EXTRACELLULAR CHITIN DEACETYLASE

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Abstract

Chitin deacetylase is an enzyme produced by several organisms, such as bacteria, fungi, and insects. In most cases, the enzyme is produced intracellularly; however, some fungi also produce the enzyme extracellularly, so the work aimed to compare the activity and the energies of activation of intracellular and extracellular chitin deacetylase. The enzymes produced by Absidia orchidis vel coerulea were used in experiments. It was shown that intracellular chitin deacetylase is significantly more active than the extracellular form. The activation energies were evaluated as 77.77 kJ/mol and 90.88 kJ/mol for intracellular and extracellular chitin deacetylase, respectively, meaning that intracellular enzymes act faster. Our paper is the first one that presents the energy of activation of chitin deacetylase. The presented energy of activation evaluation and comparison method can be used as a good tool to choose the chitin deacetylase for industrial application when the enzyme is available in different forms or comes from different sources.

Keywords: *chitin deacetylase, chitosan, energy of activation*

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1. Introduction

Chitin deacetylase, CDA (EC 3.5.1.41), is an enzyme that catalyses the hydrolysis of the acetamido groups in *N*-acetyl-D-glucosamine (GlcNAc) units of chitosan or chitin, resulting in the formation of D-glucosamine (GlcN) units:

$$-\mathrm{GlcNHAc} + \mathrm{H_2O} \xrightarrow{\text{chitin deacetylase}} -\mathrm{GlcNH_2} + \mathrm{AcOH}. \tag{1}$$

Thanks to this reaction, the acetylation degree (AD, content of GlcNAc units in a polymer chain) of the biopolymer decreases, and the activity of chitosan, especially the biological one, increases.

Chitin deacetylase is an enzyme that is produced by several organisms, such as bacteria [1, 2], fungi [3–5], and insects [6, 7]. Most enzymes are produced intracellularly, but some fungi produce the enzyme extracellularly, e.g., *Absidia coerulea* [8], *Absidia glauca*, and *Mucor rouxi* [9]. In such a case, it is important to decide which source or form of the enzyme is more suitable for industrial enzymatic deacetylation of chitosan or chitin.

The mechanism of enzymatic deacetylation of GlcNAc units can be described with the Michaelis-Menten model [10–12], according to a simple enzymatic reaction:

$$\mathrm{E} + \mathrm{S} \overset{\mathrm{K_M}}{\longleftrightarrow} \mathrm{ES} \xrightarrow{\mathrm{k_{cat}}} \mathrm{E} + \mathrm{P}. \tag{2}$$

According to that model, the enzyme (E) reacts with the substrate (S, GlcNAc unit), creating the enzyme-substrate complex (ES), which is next transformed into enzyme and product (P, GlcN). This second reaction is described as a first-order reaction, and it determines the product formation. The reaction rate constant k_{cat} is correlated with the energy of activation by the Arrhenius equation:

$$k_{cat} = k_{cat,\infty} \cdot \exp\left(-\frac{E_a}{R \cdot T}\right) \tag{3}$$

or after transformation:

$$\ln k_{cat} = \ln k_{cat,\infty} - \frac{E_a}{R} \cdot \frac{1}{T} \tag{4}$$

where:

k_{cat} – first-order reaction rate constant [1/min];
$k_{cat,\infty}$ – constant [1/min];
E_a – energy of activation [J/mol];
R – universal gas constant (8.31446 J/(mol·K));
T – temperature [K].

Thus, the activation energy can be evaluated by plotting the relationship $\ln k_{cat} = f(1/T)$. Comparing the energy of activation (E_a) of both enzyme forms, one can compare reaction rates (in the same conditions, i.e., temperature and substrate concentration). The form of the enzyme with the lower value of E_a will act faster, so it will be more suitable for industrial applications.

The work aimed to compare the activation energy of intracellular and extracellular chitin deacetylase. Additionally, a tool for choosing the chitin deacetylase for industrial application when the enzyme is available in different forms or sources will also be presented. In our investigations, enzymes produced by *Absidia orchidis* vel *coerulea* were used.

2. Materials and Methods

2.1. Chitin Deacetylase

The intracellular enzyme was separated from the biomass of *Absidia orchidis* NCAIM F 00642 (the enzyme is not commercially available). The fungi were cultivated in a 5.0 L batch culture (26°C, pH 5.5, YPG nutrient medium [13]) and separated from the nutrient medium by centrifugation (6000 rpm, 20 min). Next, the biomass was frozen, slowly thawed and homogenised, and the crude cell extract was separated by filtration (0.45 μm) and salted out with ammonium sulphate (80% saturation, 2 h). The solution was dialysed with HCl (pH 4.0) to remove ammonium sulphate (a membrane module with a cut-off of 10 kDa, Sartorius) and then concentrated by ultrafiltration (the same membrane module). This enzyme solution, adjusted with HCl to pH 4.0 (optimal pH), was used in all experiments. The concentration of the proteins assumed as the enzyme was equal to C_e = 27.03 μg/mL (C_e = 0.386 μmol/L).

The extracellular enzyme was separated from the nutrient medium of the culture of the same fungi. Culture broth was separated from biomass by centrifugation (6000 rpm, 20 min) and filtration through membrane filters (0.45 μm, cellulose nitrate). Further, the solution was concentrated by ultrafiltration using a membrane module Vivaflow 50 (the same module as given above), followed by purification based on diafiltration. The concentration of the proteins assumed as the enzyme was equal to C_e = 5.03 μg/mL (C_e = 0.072 μmol/L).

2.2. Chitosan

Chitosan from shrimps (BioLog Heppe, Germany) with a degree of acetylation (DA) of 39.2% and medium molecular weight (500 kD), according to the information of the producer, was used in all experiments. Chitosan (4.0 g) was mixed with 800 mL of pure water, then HCl solution (1%) was added dropwise under pH-controlled conditions (4.0 ± 0.1). 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 (pH 4.0). The concentration of the prepared chitosan solution was confirmed by the gravimetric method. The dilutions were obtained by mixing the concentrated solution with HCl (pH 4.0) in volumetric flasks. For kinetic studies, chitosan concentration was recalculated from mass to molar concentrations of GlcNAc (C_{GlcNAc}).

2.3. Reaction Rate

The reaction rate of the enzymatic deacetylation of chitosan was determined using the initial rate method. 50 mL of chitosan solution of a given concentration was added to a 150 mL reactor and incubated at the temperature of the reaction (35 - 50°C) for 10 min, with continuous stirring (200 rpm). 2 mL of chitin deacetylase solution (preheated at the same temperature for 2 min) was added to start the reaction. At 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 min, 500 rpm) to separate the precipitated chitosan. The supernatant was collected and filtered on syringe filters (cut-off 0.45 μm, cellulose nitrate), and the concentration of acetic acid was determined for each sample.

2.4. Analytical Methods

The concentration of acetic acid was analysed using the HPLC method, as described by Jaworska [12]. The HPLC system consisted of a HyperREZ XP Organic Acid Column and a HyperREZ XO Carbohydrate H^+ Guard Column (at 60°C). The isocratic system Varian

ProStar 210 was used with a refractometer detector Varian ProStar 350 and deionised water (0.5 mL/min) as an eluent.

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

3. Results and Discussion

The mechanism of the enzymatic deacetylation of chitosan by the enzymes produced by *Absidia orchidis* vel *coerulea* is described by the Michaelis-Menten equation:

$$\nu = \frac{k_{cat} \cdot C_e \cdot C_{GlcNAc}}{K_M + C_{GlcNAc}} \tag{5}$$

where:

ν – reaction rate [mmol/(L·min)];
k_{cat} – reaction rate constant [1/min];
C_e – protein (enzyme) concentration [mmol/L];
R – universal gas constant (8.31446 J/(mol·K));
C_{GlcNAc} – concentration of GlcNAc units [mmol/L].

The relationship between reaction rate and GlcNAc concentration was obtained for every temperature for each enzyme. Then, experimental data were plotted using the Lineweaver-Burk transformation ($(1/\nu)$ vs $(1/C_{GlcNAc})$). For that plot, linear relationships were obtained for all series of experiments (the R^2 was in the range from 0.91 to 0.98), and the k_{cat} values were evaluated based on the intercept with the Y-axis (Table 1).

Table 1. The kinetic parameters of the Michaelis-Menten equation for different temperatures.

Temperature		**k_{cat} [1/min]**	**$k_{3\ intracel}$ [1/min]**
[°C]	**[K]**		
35	308.15	-	251.7
40	313.15	0.026	342.9
45	318.15	0.045	620.8
50	323.15	0.029	990.1

Based on the data obtained, it can be stated that the intracellular chitin deacetylase is significantly more active than the extracellular enzyme. The reaction rate constant values differ by at least three orders of magnitude. Additionally, at the temperature of 35°C, the activity of the extracellular enzyme was so low that the data obtained were below the detection level. The decrease in the observed k_{cat} values for the 50°C temperature may be caused by thermal deactivation (the reaction rate constant decreased nearly two times compared to data obtained at 45°C).

In the next step, the energies of activations were evaluated using Equation 4, with the slope ($-E_a/R$) extracted, as presented in Figure 1.

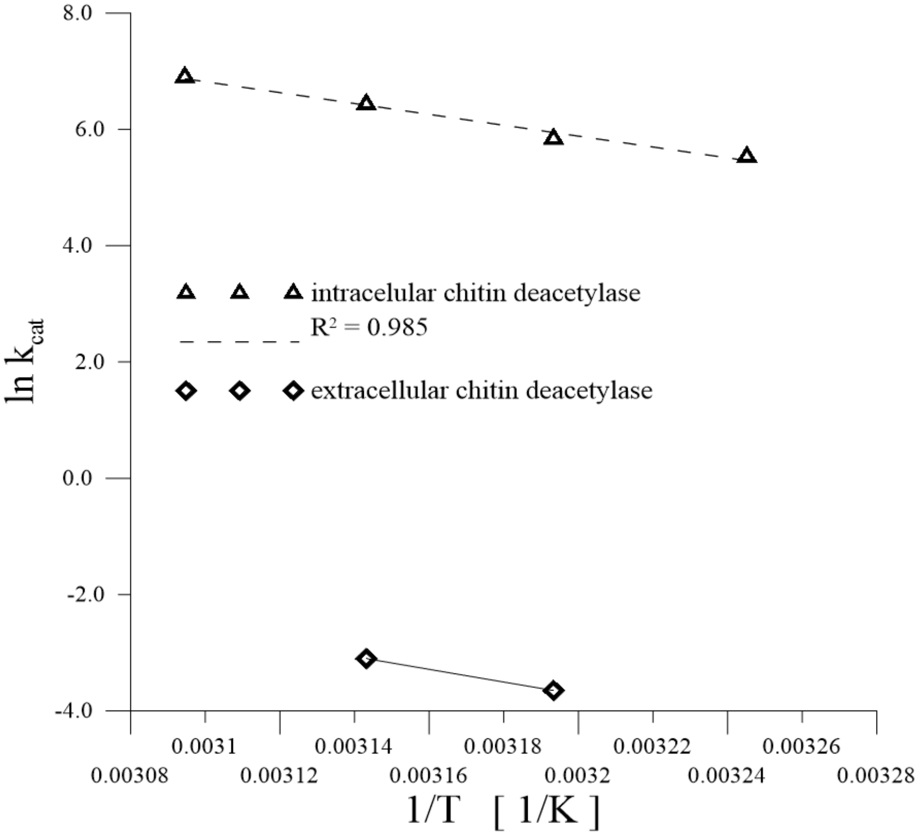

Figure 1. The Arrhenius plot for intracellular and extracellular chitin deacetylase.

Based on data presented in Figure 1, the activation energies were evaluated as 77.77 kJ/mol and 90.88 kJ/mol for intracellular and extracellular chitin deacetylase, respectively. These values confirm that intracellular chitin deacetylase is more active, as the activation energy for that enzyme is lower.

4. Conclusions

The activation energy is a barrier that reagents must pass to react. The catalyst, including enzymes, lowers this barrier; thus, the reaction goes faster. However, when several catalysts can be used, one has to choose a proper one, usually by comparing the activation energies. Chitin deacetylase is an enzyme that can be produced intracellularly or extracellularly by several different organisms. For industrial applications, one must decide which enzyme source should be used. Our investigations present a valuable tool for choosing the proper enzyme source.

In the present experiments, chitin deacetylases produced by *Absidia orchidis* vel *coerulea* were compared. We showed that intracellular chitin deacetylase is significantly more active than the extracellular form. Both enzymes differ not only in activity but also in the values of activation energies. E_a for intracellular enzymes is lower, which means that intracellular enzymes will act faster.

This paper is the first one that presents the energy of activation of chitin deacetylase; thus, comparison of data with others in the literature was not possible.

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