

# **pH STABILITY AND KINETICS OF pH DEACTIVATION OF CHITIN DEACETYLASE PRODUCED BY *Absidia orchidis***

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## **Abstract**

*This paper presents the influence of pH on the activity and stability of an extracellular chitin deacetylase produced by *Absidia orchidis*. It showed the highest activity at pH 4.0 and was most stable at pH 9.5. In addition, there was pH deactivation that could be described by first-order irreversible kinetics. The pH deactivation rate constant changed linearly with pH in the range from 4.0 to 9.0.*

**Keywords:** *chitin deacetylase, pH-deactivation, pH-stability*

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## 1. Introduction

The optimal pH for an enzyme's activity represents important information because it influences the conditions used for the industrial application of an enzyme. The changes in an enzyme's activity with pH are usually connected with changes in its tertiary conformation and, thus, changes in the conformation of the active centre. Changes in the tertiary conformation of an enzyme are the result of the interaction between its amino acids and the environment. Its optimal conformation at the optimal pH results in the highest activity.

The optimal pH of chitin deacetylase (CDA, EC 3.5.1.41), the only enzyme that deacetylates *N*-acetyl glucosamine units (GlcNAc in chitin and chitosan, depends on the source of the enzyme. For intracellular CDA, the optimal pH is in the range from 4.0 (*Absidia orchidis* [1]) to 5.8 (*Mucor rouxii* [2]), while for extracellular CDA, the optimal pH ranges from 7.0 (*Aspergillus nidulans* [3]) to 12.0 (*Colletotrichum lindemuthianum* [4]). In addition to the influence of pH on the activity of CDA, the pH stability and pH deactivation of this enzyme should be investigated. These parameters are of particular importance due to the desirable industrial applicability of CDA; therefore, it is necessary to determine the pH that ensures the highest CDA activity and the pH that results in the best stability during long-term storage. One must remember that acetic acid is liberated during deacetylation, so changes in the pH of the reaction solution are possible. The present paper aimed to investigate the influence of pH on CDA activity, its pH stability and pH deactivation.

## 2. Materials and Methods

### 2.1. Chitin Deacetylase

CDA is not commercially available, so it was partially purified from the culture medium of the fungus *A. orchidis* NCAIM F 00642 (late logarithmic growth phase). This fungus was cultivated on YPG nutrient medium at 26°C and pH 5.5 in a batch culture, according to Jaworska and Konieczna [5]. Culture broth was separated from biomass by centrifugation (3300 g, 10 min) and filtration on membrane filters (0.45 µm, cellulose nitrate). The solution was purified by ultrafiltration using membrane module Vivaflow 50 (Sartorius, cut-off of 30 kDa) followed by diafiltration (with the same module) in hydrochloric acid (HCl, pH 4.0). The enzyme solution was stored at 4°C. The activity (A) of CDA (molecular weight = 70 kDa) was defined as the amount of enzyme that can increase the concentration of acetic acid by 1 nmol/ml during 1 min [1U = (nmol/ml/min)]. When indicated, the activity is expressed as specific activity ( $A_{sp}$ ): U/mg [(nmol/ml)/(min × mg of protein)].

### 2.2. Chitosan

Chitosan from shrimp (BioLog Heppe, Germany) with a degree of acetylation of 23% and a medium molecular weight (the viscosity of a 1% solution in 1% acetic acid was 200 mPa), according to the information from the supplier, was used in all experiments. Chitosan (4.0 g) was mixed with 800 ml of pure water, and 1% HCl was added dropwise under pH-controlled conditions ( $4.0 \pm 0.1$ ). The solution was filtered on paper filters with a cut-off of 1 µm to remove insoluble residues. The volume was adjusted to 1000 ml with HCl (pH 4.0). The concentration of the prepared chitosan solution was confirmed by the gravimetric method.

### **2.3. Reaction Rate**

The rate of enzymatic deacetylation of chitosan was determined using the initial rate method. Twenty-five millilitres of chitosan solution was added to a 50-ml reactor and incubated at 45°C for 15 min, with continuous stirring (200 rpm). Two millilitres of CDA solution were added to start the reaction. At appropriate time intervals, 2-ml samples were collected and mixed immediately with 0.1 ml of 1 M sodium hydroxide (NaOH) to stop the reaction. The samples were centrifuged (3300 g, 10 min) to separate the precipitated chitosan. The clear supernatant was collected, and the acetic acid concentration was determined.

### **2.4. Effect of pH on the Activity and Stability of Chitin Deacetylase**

The influence of pH on CDA activity was measured using the method described in Section 2.3, where 25 ml of chitosan solution with a pH of 3.0–6.0 was used. The pH of the chitosan solution was set at a specified level using 0.1 M Tris or 1% HCl before CDA was added. The pH of the CDA solution was identical to the reaction mixture and adjusted just before the reaction. The reaction was initiated by adding 2 ml of the CDA solution to the chitosan solution.

For the pH stability evaluation, the CDA solution was divided into fractions, and each fraction was set at a defined pH. The samples were stored for 7 days at 5–8°C in a fridge. The CDA activity was evaluated according to the method described in Section 2.3, using 25 ml of a chitosan solution at pH 4.0. The reaction was initiated by adding 2 ml of the CDA solution. The initial CDA activity (just after isolation, at pH 4.0) was determined and treated as a reference value for each fraction.

### **2.5. Analytical Methods**

The acetic acid concentration was determined using high-performance liquid chromatography (HPLC), as described by Jaworska [1]. The HPLC system consisted of a HyperREZ XP Organic acid column and HyperREZ XO Carbohydrate H<sup>+</sup> Guard Column (at 60°C). A Varian ProStar 210 isocratic system was used with a Varian ProStar 350 refractometer detector and 0.0025 M sulfuric acid (H<sub>2</sub>SO<sub>4</sub>, 0.5 ml/min) as an eluent.

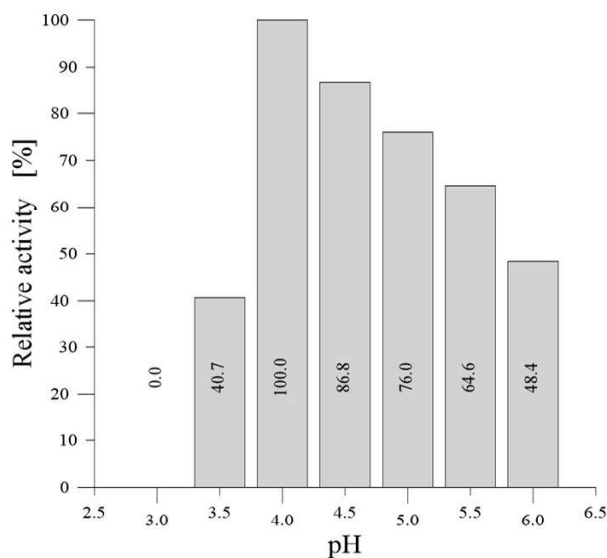
The protein concentration was measured using Bradford's method [6] with Coomassie Brilliant Blue (Bio-Rad, USA). The calibration curve was prepared using bovine serum albumin as a standard.

## **3. Results and Discussion**

*A. orchidis* synthesises intracellular and extracellular CDA [7]. In our earlier experiments, we used intracellular CDA. In the present study, extracellular CDA was used because it is easy to isolate.

### **3.1. Optimal pH and pH Stability**

The activity of CDA was investigated in the pH range from 3.0 to 6.0 (Figure 1). The activity of CDA in neutral pH was very low or even absent in alkaline pH due to precipitation of chitosan; hence, it is not presented in Figure 1.



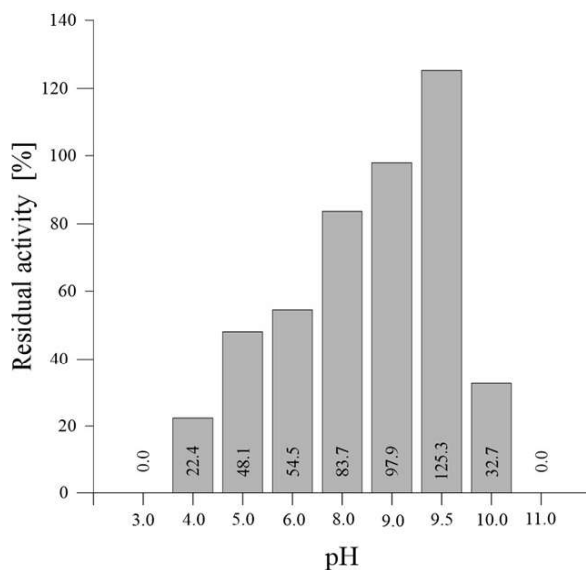
**Figure 1.** The influence of pH on the activity of chitin deacetylase; the specific activity was 422.8 U/mg at pH 4.0 and 45°C.

The highest activity was observed at pH 4.0 (422.8 U/mg). Of note, CDA was more sensitive to a decrease from this optimal pH to more acidic conditions than an increase to more basic conditions. At pH 3.5, the activity was almost 60% lower than at pH 4.0, and at pH 3.0, CDA was completely inactive. At pH 4.5, CDA's activity was over 90% of that for the optimal pH, and it showed a 10%–15% drop in activity with each 0.5 increase in pH, up to pH 6.0. The experiments were not carried out at higher pHs as chitosan precipitated, and the data would have been difficult to compare due to the different forms of substrate used.

The extracellular CDA used in this study had an optimal pH of 4.0. The optimal pH for extracellular chitin deacetylase has usually been reported in the neutral or alkaline range: pH 7.0 for *A. nidulans* CECT 2544 [3], pH 11.0–12.0 for *C. lindemuthianum* ATCC 56676 [4] and pH 7.5 for *Scopulariopsis brevicaulis* [8]. On the other hand, intracellular enzymes usually have an optimal pH that is slightly acidic – for example, pH 4.5 for the enzyme separated from *M. rouxii* [9] and pH 5.0 for the enzyme separated from *Absidia coerulea* [10]. However, an optimal pH in the alkaline range for extracellular chitin deacetylase is not a rule. Win and Stevens [7] performed experiments with extracellular chitin deacetylase secreted by *A. orchidis* in phosphate buffer with a pH of 5.8. In addition, a chitin deacetylase secreted by *Rhizopus circinans* also had an optimal pH in the acidic range (pH 5.5–6.0) [11].

The pH stability of an enzyme is especially important when it works in an environment that is acidified or alkalinised during the reaction. Additionally, pH stability may play an important role in purification processes where solutions with a pH different from the enzyme's optimal pH are used (e.g. chromatographic purification).

The stability of CDA at different pHs was assessed for 7 days at 5–8°C to avoid thermal deactivation. After storage, the activity of CDA was measured in optimal conditions (pH 4.0) and compared with the initial activity (pH 4.0), which was evaluated just after purification. The changes in CDA activity are presented in Figure 2.



**Figure 2.** The activity of chitin deacetylase after 7 days of storage at the indicated pH. The initial specific activity was 422.8 U/mg at pH 4.0 and 45°C.

The results showed that CDA was stable in alkaline but not acidic (optimal pH for the reaction) storage conditions. The samples stored at pH 9.5 had the highest activity (125.3% of the initial value), while the samples stored at the optimal pH (4.0) showed activity that decreased to 22% of the initial value. Increasing the storage pH above 9.5 markedly decreased CDA activity: at pH 10.0, it was three times lower than the initial value, and at pH 11.0, CDA was inactivated. This study is the first to report the influence of the storage pH on CDA activity.

### 3.2. Kinetics of pH Deactivation

Enzymes can be deactivated due to several factors, including high temperature and pH. An enzyme's pH stability and the kinetics of pH deactivation are not often reported in the literature. Based on the pH stability results, the kinetics of pH deactivation was evaluated. The process was assumed to follow first-order irreversible deactivation. This mechanism is described by equation (1):

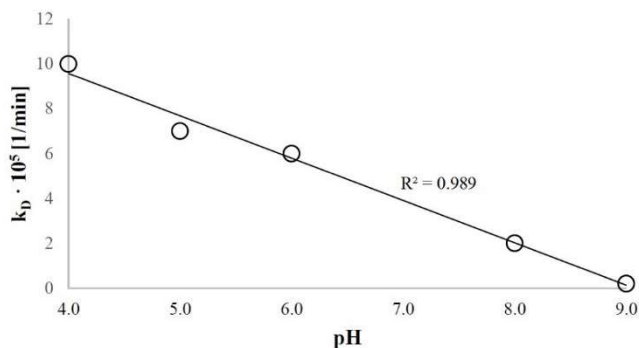
$$\frac{dU}{dt} = -k_{D,pH}U \quad (1)$$

where  $U$  is the enzyme activity [U/mg],  $t$  is time [min] and  $k_{D,pH}$  is the pH deactivation constant [1/min].

Integration with the initial conditions  $U = U_0$  for  $t = 0$  yields equation (2):

$$\ln \frac{U}{U_0} = -k_{D,pH}t \quad (2)$$

Based on the linear correlation  $\ln(U/U_0)$  versus  $t$ ,  $k_{D,pH}$ , the deactivation constant for each pH was evaluated. The dependence of pH on  $k_{D,pH}$  is presented in Figure 3. The data point for pH 9.5 was not considered because there was activation rather than deactivation.



**Figure 3.** The dependence of the deactivation constant ( $k_{D,pH}$ ) on pH.

The results showed that  $k_{D,pH}$  decreased linearly as the pH of the storage solution increased from 4.0 to 9.0. The relation can be approximated by the linear correlation shown in equation (3):

$$k_D = (-1.89 \times 10^{-5}) \times (\text{pH}) + (17.13 \times 10^{-5}) \quad (3)$$

The value of  $k_{D,pH}$  is the highest at optimal pH (4.0), which means that the deactivation is the fastest at that pH, while the deactivation rate constant is the lowest for pH 9.0, which means that the enzyme is stable at that pH.

#### 4. Conclusions

Based on the results, pH 4.0 is the optimal pH for the enzymatic deacetylation of chitosan by extracellular CDA of *A. orchidis*. This optimal pH differs from what has been reported for other extracellular enzymes, which are usually in the neutral or alkaline range (pH 7–12). Contrary to our expectations, CDA was more stable in alkaline conditions (pH 9.0–9.5) than in acidic conditions (pH 4.0). High short-term pH stability in neutral or alkaline solutions has also been reported for extracellular chitin deacetylase produced by *Mortierella* sp. [12], *Absidia corymbifera* [13], *Nitratireductor aquimarinus* [14] and *Microbacterium esteraromaticum* [15]; however, contrary to the findings in the present study, those enzymes showed the highest activity in the same pH range. The reason why extracellular CDA from *A. orchidis* shows the highest stability in alkaline conditions but the highest activity is in acidic conditions is unknown and difficult to explain and requires further investigation. Investigation of pH-dependent deactivation showed that the deacetylation process can be described by first-order irreversible kinetics, where the deactivation constant is a linear function of pH in the range from 4.0 to 9.0. The present study represents the first-time results related to pH deactivation and long-term storage pH conditions for any of the chitin deacetylases.

#### 5. Acknowledgement

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