Kinetics of enzymatic deacetylation of chitosan

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Abstract The current paper reports on an investigation of the kinetics of chitosan deacetylation by chitin deacetylase isolated from *Absidia orchidis* vel *coerulea*. The reaction rate was correlated with the concentration of GlcNHAc units of the polymer. It is shown that the process follows the Michaelis–Menten mechanism. Modification of the Michaelis–Menten equation by introducing the activity of the enzyme instead of its concentration was tested and found to give a better approximation to the experimental data than the original Michaelis–Menten model. Parameters for both the original and the modified Michaelis–Menten equations are also proposed.

Keywords Chitin deacetylase · Chitosan · Enzyme activity · Modified Michaelis–Menten equation · Kinetics of impure enzyme preparation · Nonhomogeneous preparation

Introduction

Chitin deacetylase is the only enzyme that is able to hydrolyze the linkage between the acetyl and amine groups in the units of *N*-acetylglucosamine

(GlcNHAc) of chitin or chitosan, transforming them into the glucosamine (GlcNH₂) units according to the reaction:

This transformation can be used for enzymatic modification of chitosan to obtain polymers with a lower degree of acetylation (DA, the content of GlcNHAc in a polymer chain), as several properties of chitosan (e.g. bioactivity, biodegradability, sorption capacity) are related to acetylation degree, varying with variation in DA. The reaction of deacetylation can be carried out chemically with concentrated NaOH solution (approx. 50%, 90–120 °C), but this causes simultaneous degradation of the chitosan chain, so the polymer can be degraded even to oligomers. Contrary to the chemical process, enzymatic deacetylation avoids polymer degradation and a polymer with the same degree of polymerisation and much smaller acetylation degree is obtained.

Chitin deacetylase (ChD) exists as intracellular enzyme (e.g. from *Mucor rouxi*, *Absidia orchidis*) or as extracellular enzyme (e.g. produced by *Colletotrichum lindemuthianum*, *Aspergillus nidulans*). The mode of action of these two forms of chitin deacetylase is different. It was suggested that for extracellular ChD it is a “multiple chain mechanism” while for intracellular ChD it is a “multiple attack” mechanism and the deacetylation starts from nonreducing end of chitin/chitosan chain (Tsigos et al. 2000; Blair et al. 2006).

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Industrial application of an enzyme needs knowledge of the kinetics of the process. Currently the literature shows several possible kinetic models for enzymatic deacetylation of chitosan. It has been suggested that the process with chitin deacetylase separated from *Mucor rouxi* follows the Michaelis–Menten mechanism (Martinou et al. 1998) but, depending on the degree of acetylation of the polymer, different values of K_M were reported and values of V_{max} (or k_3) were not presented. Contrary to these observations, Dunkel and Knorr (1994), Amorim et al. (1996) as well as Jaworska and Konieczna (2003) have suggested some deviations from the Michaelis–Menten mechanism. Dunkel and Knorr (1994) did not observe the saturation-type curve for the enzyme isolated from *Mucor rouxii* DSM 1191, so they concluded that the kinetics are not of the Michaelis–Menten type. Amorim et al. (1996) suggested that the kinetics for chitin deacetylase isolated from *Cumin-gamells bertholletiae* followed the Hill affinity distribution rather than a Michaelis–Menten mechanism, probably showing allosteric behaviour. Similar observations were reported in the preliminary investigations of Jaworska and Konieczna (2004) for the enzyme isolated from *Absidia orchidis* vel *coerulea* NCAIM F0642. Data presented in the form of a Lineweaver–Burk plot did not give a linear relationship, but linearity was obtained for the plot of $1/(\text{reaction rate})$ versus $1/(\text{concentration})^2$ that could suggest a multi-site cooperative mechanism often described by the Hill's equation. The literature also presents kinetic data for enzymatic deacetylation of chitin oligosaccharides (Tokuyasu et al. 1996; Alfonso et al. 1995). Although authors have suggested the Michaelis–Menten mechanism for the deacetylation process, they reported that changes in values of V_{max} (or k_3) and/or K_M were observed with changes in the degree of polymerisation of the oligomers ($DP = 2\text{--}6$) (Table 1). Hence these parameters cannot be applied directly to the deacetylation of chitosan.

The literature data to date indicates that the kinetics of enzymatic deacetylation of chitosan has not been presented in a satisfactory way. Several kinetic models for the process have been considered: simple enzymatic kinetics according to the Michaelis–Menten model; the model of allosteric interaction; or a model of multisite cooperation. Additionally the parameters of the models changed with a change in the substrate: either with a change of degree of polymerization

(oligomers) or with a change in the degree of acetylation (chitosan). This situation suggests that every chitosan substrate would require a new set of K_M and V_{max} (k_3) values depending on the degree of acetylation, hence none of the previously reported sets of parameters can be used as universal ones suitable for deacetylation of any chitosan.

The aim of the current work was to investigate the kinetics of deacetylation of chitosan and to present a procedure for evaluation of kinetics parameters that would be suitable for chitosans with different acetylation degree. In this work, chitin deacetylase separated from *Absidia orchidis* v. *coerulea* NCAIM F 00642 was used.

Materials and methods

Chitin deacetylase

Chitin deacetylase was separated from *Absidia orchidis* v. *coerulea* NCAIM F 00642. The fungi were cultivated in a 7.0-L batch culture (26 °C, pH 5.5, YPG nutrient medium (Jaworska and Konieczna 2001)) and separated from the nutrient medium by centrifugation (6,000 rpm). Next the biomass was frozen and then slowly thawed and homogenised, and the crude cell extract separated (centrifugation, 6,000 rpm) and salted out with ammonium sulphate (80% saturation) overnight at 4–6 °C. The solution was diafiltrated with HCl (pH 4.0) to remove ammonium sulphate (using a PES membrane module Vivaflow 50 (Sartorius) with a 10 kDa cut-off) and then concentrated by ultrafiltration (the same membrane module). This solution was used in the experiments. The enzyme was accompanied by one additional protein (SDS electrophoresis not shown) with a trace concentration (as indicated by a much lower intensity of the bar) that stabilised its activity during storage.

The presence of chitosanolytic enzymes was monitored by viscometric measurements and reducing sugar concentration measurements, but their activity was not observed (changes in the range of accuracy of analytical methods were detected, and they did not exceed 5% of initial values).

Enzyme activity was defined as the amount of enzyme that releases 0.1 mg of acetic acid during 1 min of the reaction at optimal conditions (pH = 4.0, T = 50 °C and with concentration of GlcNHAc

Table 1 Kinetic parameters of the Michaelis–Menten equation

Source	Substrate	K _M	V _{max}	Ref.
<i>Colletotrichum lindemuthianum</i> ATCC 56676	(GlcNAc) ₂	18.4 mM	16.2*	Tokuyasu et al. (1996)
	(GlcNAc) ₃	11 mM	11.3*	
	(GlcNAc) ₄	0.6 mM	184*	
	(GlcNAc) ₅	0.4 mM	158*	
	(GlcNAc) ₅	69.9 μM	–	
<i>Aspergillus nidulans</i> CECT 2544	(GlcNAc) ₂	0.09 mM	4.0**	Alfonso et al. (1995)
	(GlcNAc) ₃	0.15 mM	5.3**	
	(GlcNAc) ₄	0.50 mM	10.0**	
	(GlcNAc) ₅	2.50 mM	33.3**	
	(GlcNAc) ₆	0.12 mM	5.9**	
<i>Mucor rouxii</i> ATCC 24905	Chitosan AD 8%	2.1 mg/mL	–	Martinou et al. (1998)
	Chitosan AD 35%	1.7 mg/mL	–	
	Chitosan Ad 62%	2.1 mg/mL	–	

* μM GlcN/(min*mg białka)

** nmol/min

close to saturation, $C_{\text{GlcNAc}} = 2.26 \text{ g/L}$, $1 \text{ U} = 0.1 \text{ mg/min}$.

Chemicals

The chitosan used in all the experiments was kindly donated by Gillet–Mahtani–Chitosan (France/India). It was of medium molecular weight (viscosity of 1% solution in 1% acetic acid solution at 25 °C $\mu = 85 \text{ Pa} \times \text{s}$, according to the producer data), and AD = 39.8% (determined from the IR spectrum using the procedure of Domszy and Roberts (Domszy and Roberts 1985)). Chitosan (5.0 g) was mixed with 500 mL of HCl solution (pH 4.0). Next 0.1 M HCl was added in small portions (1–2 mL) to complete polymer dissolution (the pH was controlled during the dissolution and kept constant at $\text{pH } 4.0 \pm 0.1$) and it was adjusted to a final volume of 1.0 L with a HCl solution (pH 4.0). The lower concentration of GlcNAc unit in reaction mixture was obtained by dilution of prepared solution with HCl (pH 4.0) in a volumetric flask.

All other chemicals were analytical grade and purchased from POCH (Poland).

Kinetics experiments

100 mL of the chitosan solution with the required concentration of GlcNAc units, prepared as described above, were preheated (50 °C) for 20 min and the chitin

deacetylase solution was preheated separately for 2 min at 50 °C. The reaction was initiated by adding the enzyme to the reaction solution and was continued at 50 °C in a stirred (250 rpm) thermostated batch reactor. At preselected time intervals the reaction mixture was sampled (2.0 mL) and the reaction was stopped immediately by addition of 0.10 mL 1.0 M NaOH to the sample. The precipitated chitosan was separated by centrifugation and the released acetic acid concentration in the clear supernatant solution was determined.

The reaction rate in each experiment was calculated as an initial reaction rate on the basis of the changes of the concentration of acetic acid in time.

Analytical methods

Protein concentration was determined according to the Bradford method using a ready-made reagent from Biorad (USA, cat. No. 500-0006) and bovine serum albumin as a standard.

Acetic acid concentration in the clear solution was analyzed by the HPLC method using an isocratic system (Varian ProStar 210) with a HyperREZ XP Organic acid column (60 °C) and a HyperREZ XO Carbohydrate H⁺ Guard Column, 0.0025 M H₂SO₄ as eluent (0.5 mL/min), and a refractometer detector (Varian ProStar 350). The quantification limit was evaluated at 5 nmol/mL with a standard deviation of 8% of the mean value.

The method was validated for acetic acid determination in chitosan-HCl (pH 4.0) solutions.

Results

Experiments were carried out for 5 different concentrations of enzyme, and 8–9 concentrations of chitosan for each concentration of the enzyme. Four series of experiments were used for evaluation of the parameters in a kinetics equation while the fifth was used as independent data for verification of the proposed kinetics equation.

Experiments were carried out at optimal pH (4.0) and at temperature of 50 °C which was 5 °C lower than the optimal. The initial reaction rate (v , [(mg AcOH/L)/min]) of enzymatic deacetylation was correlated with the concentration of GlcNAc units as a substrate (their concentration, C_{GlcNAc} , being calculated on the basis of the chitosan concentration and its degree of deacetylation) as it was presented earlier (Jaworska et al. 2009). Using this concept, a simple, universal relationship can be obtained which can be applied to any chitosan with a known DA value without need to introduce any additional parameters to correct the particular DA value of the polymer.

Results of the enzymatic deacetylation of chitosan are presented as a Lineweaver–Burk plot in Fig. 1.

In all cases the linearity between reverse reaction rate and reverse concentration can be readily observed. This linearity has been confirmed also by an Eadie–Hofstee plot and a Wolf plot (data not presented). The coefficients of determination (R^2) varied from 0.962 to

0.968 what indicate a good approximation to experimental data. On the basis of these observations, the agreement with the Michaelis–Menten model was assumed. Although the mechanism itself was not investigated, deviations from the Michaelis–Menten model were not observed and this equation has been chosen for the mathematical model of enzymatic deacetylation of chitosan as it has a simple, elegant form and describes the experimental data with a sufficient accuracy.

$$v_{\text{AcOH}} = \frac{V_{\text{max}} \cdot C_{\text{GlcNAc}}}{K_{\text{M}} + C_{\text{GlcNAc}}} = \frac{k_3 \cdot C_{\text{E}} \cdot C_{\text{GlcNAc}}}{K_{\text{M}} + C_{\text{GlcNAc}}} \quad (1)$$

The parameters in Michaelis–Menten equation have been evaluated on the basis of the original experimental data (v_{AcOH} vs. C_{GlcNAc}) using the nonlinear regression method. The parameters of the Michaelis–Menten equation are presented in Table 2.

The presented data show that the variation in the Michaelis constant (K_{M}) is small and acceptable, but large differences in the reaction rate constant (k_3) are observed; with the highest value (0.876) being nearly 90% larger than the lowest value (0.477). Such significant differences for the calculated values of k_3 cannot be accepted for a kinetic model. It can be easily observed when we compare experimental and theoretical data calculated on the basis of the mean values of K_{M} and k_3 , Fig. 2.

The proposed model, based on the mean values presented in Table 2, describes only two experimental series well (Fig. 2B), while showing significant differences for the other two experimental series (Fig. 2A). It also shows significant differences for the

Fig. 1 The Lineweaver–Burk plot for the enzymatic deacetylation of chitosan

Fig. 2 Comparison of experimental data with the model (Eq. 1). **A**, **B** experimental data showed in Fig. 1; **C** data for additional experiment. The mean values of K_M and k_3 (Table 2) were used for calculation of the model

Table 2 Parameters of the Michaelis–Menten equation, according to Eq. 1

C_E (mg/L)	K_M (mg/L)	k_3 (1/min)
0.822	693	0.477
1.442	532	0.577
2.884	533	0.876
3.605	750	0.737
Mean $\pm$ SD	627 $\pm$ 111	0.667 $\pm$ 0.176

experimental results used for the model verification (Fig. 2C). Such large differences are not acceptable in kinetic studies.

Chitin deacetylase is an enzyme that is not commercially available and it was separated from the biomass in laboratory conditions, so preparations with different initial activity could be obtained. It was also not purified to homogeneity because the homogeneous enzyme preparation showed a very short stability and lost its activity in 1–2 days, while enzyme preparations with some amount of impurities were stable for 2–3 weeks when stored under refrigerated conditions. The impurities present in the non-homogeneous preparation stabilized its activity.

In such a case, when operating with preparations with different initial activity and not purified to homogeneity, the kinetics studies become extremely complicated. The concentration of enzyme (C_E) cannot be used in the kinetic equation as the real C_E is often not known. The concentration of proteins in a

preparation (usually used as C_E) also contains an unknown concentration of impurities. Thus, instead of using the concentration of the enzyme it was decided to correlate the reaction rate with the “concentration of activity” (U/L), as activity is correlated only with the enzyme taking part in the reaction. The maximal reaction rate, V_{max} , is the parameter that depends on enzyme concentration. Thus the maximal reaction rate V_{max} can be modified:

$$V_{max} \sim C_E \sim \frac{U}{L}. \quad (2)$$

The “concentration of activity” can be calculated on the basis of the specific activity (U/mg) of the preparation and the concentration of proteins (C_p) in it:

$$\frac{U}{L} = \frac{U}{mg} \times C_p = \left(\frac{U}{mg} \right) \times \frac{mg}{L}. \quad (3)$$

Data presented in Fig. 1 indicated that the kinetics of the enzymatic deacetylation of chitosan can be described by the Michaelis–Menten equation, thus this relationship was used in the modified form:

$$v_{AcOH} = \frac{\kappa \cdot \left(\frac{U}{mg} \right) \cdot C_p \cdot C_{GlcNAc}}{K_M + C_{GlcNAc}}. \quad (4)$$

Here, the reaction rate constant (k_3 , min^{-1}) was replaced by the κ parameter (unitless) because the activity itself contains time in its units (1 U = [mg/min]), so κ can not be considered as a rate constant. It should be emphasized that the usage of “concentration of activity” does not change the mechanism of the

Fig. 3 Comparison of experimental data with the model (Eq. 4). **A, B** experimental data showed in Fig. 1; **C** data for additional experiment. The mean values of K_M and κ (Table 3) were used for calculation of the model

Michaelis–Menten model, but changes only the way the enzyme concentration is introduced into the equation.

The parameters of the modified Michaelis–Menten equations were calculated on the basis of the original experimental data using the method of non-linear regression, Table 3.

The value of the Michaelis constant K_M remained the same, but much smaller variations in values of the parameter κ can be readily observed. The difference between the largest and the smallest calculated value was approximately 18% of the smallest value. Such difference, in our opinion, is acceptable in kinetic experiments. The mean values of K_M and κ presented in Table 3 were used to calculate the model of enzymatic deacetylation of chitosan that was compared with the experimental data, Fig. 3.

The modified Michaelis–Menten model fits the experimental data much better than the model calculated on the basis of the original equation.

Conclusions

The Michaelis–Menten equation, although having several disadvantages, is the most popular equation describing the kinetics of enzymatic reactions. It is simple and usually reflects the experimental data well. However when the initial activity of the enzyme preparation is not repeatable, significant differences

Table 3 Parameters for the modified Michaelis–Menten equation, according to Eq. 4

C_p (mg/L)	K_M (mg/L)	U units	κ
0.822	693	3.67	0.1301
1.442	532	4.68	0.1231
2.884	534	8.54	0.1187
3.605	750	6.66	0.1107
Mean $\pm$ SD	627 $\pm$ 111	–	0.1207 $\pm$ 0.0082

between data and model can be observed. These differences will be greater when the enzymatic preparation contains impurities or when using preparations with different activities. To eliminate these disadvantages it is proposed to correlate V_{max} with the activity of the enzyme instead of with its concentration. This modification has been found to give a much better approximation of the experimental data than did the original equation.

The kinetics of the deacetylation of chitosan by chitin deacetylase has not previously been described satisfactorily. Some earlier kinetic data suggested Michaelis–Menten kinetics, but the kinetic parameters would be difficult to transfer into other experiments. The current investigation confirms that the Michaelis–Menten model can be used for enzymatic deacetylation of chitosan and the evaluated parameters can be easily transferred to any chitosans used in further experiments.

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