CHEMICAL ENGINEERING TRANSACTIONS Volume 24, 2011
Editor Sauro Pierucci
Copyright © 2011, AIDIC Servizi S.r.l., ISBN 978-88-95608-15-0 ISSN 1974-9791
DOI: 10.3303/CET1124147

Kinetics of Hydrolysis Reaction of Chitin Oligomer by a Chitinase Purified from *Bacillus subtilis*

Kazuo Kondo*, Yu Hiyama, Michiaki Matsumoto

Department of Chemical Engineering and Materials Science
Doshisha University, Kyotanabe-city, Kyoto 610-0321, Japan
kkondo@mail.doshisha.ac.jp

Chitinases were extracted and purified from *Bacillus subtilis*, a bacterium generated from natto, which is a traditional Japanese food. First of all, the best culture condition, culture medium and culture time were decided. Secondly, the crude extracelluler enzyme was obtained by filtration and purified by ion exchange chromatography. As the result, three major fractions with high chitinase activity were obtained and those were named as Chi-1~3. The molecular weights of Chi-1~3 were 85, 30 and 55 kDa, respectively. The optimum pH and the optimum temperature of Chi-1~3 were obtained. For Chi-1~3, the maximum reaction rate and the Michaelis constant were obtained for the hydrolysis of glycol chitin. Chi-3 was found to hydrolyze *N*-acetyl-chito-oligosaccharides $(GlcNAc)_n$ as follows; $(GlcNAc)_5 \rightarrow (GlcNAc)_3 + (GlcNAc)_2$, and $(GlcNAc)_4 \rightarrow 2(GlcNAc)_2$.

1. Introduction

Chitin, which is a liner polymer of β-1, 4-linked *N*-acetyl-D-glucosamine (GlcNAc), is one of the most abundant biopolymers in nature (Chitin and chitosan research group, 1995). Chitinases are the enzymes hydrolyzing chitin and play an important role in ecological field. Chitin oligomer, that is, *N*-acetyl-chito-oligosaccharides, which are produced from chitin, are being paid practical attention as new food material. At present, *N*-acetyl-chito-oligosaccharides are mainly made from chitin by using hydrochloric acid. However, this method has some disadvantageous effects. Therefore, the production of *N*-acetyl-chito-oligosaccharides by enzyme is now being researched. This enzyme method does not have disadvantageous effects in environment.
Bacteria liberate some chitinases to hydrolyze the chitinolytic materials when they are growing. A number of chitinases have been reported in various bacteria from sea and soil (Chitin and Chitosan research group, 2004). Among these, *Bacillus subtilis* is a famous chitinase-producing bacterium. In this study, we tried induction and purification of chitinases from *Bacillus subtilis*, a bacterium generated from natto, which is a traditional Japanese food. We analyzed the chitin hydrolysis reaction by Chi-3, which has the greatest activity among the three purified chitinases.

2. Experimental

2.1 Enzyme and Protein Assays

Chitin powder from crab shell of reagent grade was purchased. Glycol chitin was prepared from the chitin according to the conventional method (Chitin and chitosan

Please cite this article as: Kondo K., Hiyama Y. and Matsumoto M., 2011, Kinetics of hydrolysis reaction of chitin oligomer by a chitinase purified from bacillus subtilis, Chemical Engineering Transactions, 24, 877-882
DOI: 10.3303/CET1124147

research group, 1995). Chitin oligomer, that is, *N*-acetyl-chito-oligosaccharides ($(GlcNAc)_n$, n=2 to 5) were also purchased.
Chitinase activity was determined by a modified Schales method (Imoto and Yagishita, 1971) using glycol chitin as the substrate. In the modified Schales method, the chitinase activity is defined as the difference in absorbance at 418.5 nm between sample and blank solutions. This is hereafter described as ΔABS. at 418.5 nm. The protein concentration was determined by the Bradford method with bovine serum albumin as a standard (Fukui, 1991).

2.2 Cultivation of Microorganism and Effect of Cultivation Time on Activity

Bacillus subtilis 1076 was purchased from IAM Culture Collection, and was cultivated in a liquid medium. For pre-cultivation, carbon sources used were glucose and GlcNAc. For main cultivation, carbon sources were colloidal chitin and chitin.
After the main cultivation for 24 h, the multiplied bacteria and the liquid medium were separated from each other by filtration. Crude extracellular enzyme was obtained by concentration and dialysis of the filtrate. The induction of chitinase was tried under some culture conditions in the main cultivation. After starting the main cultivation, 20 ml sample was collected at designated cultivation time. The crude extracellular enzyme was obtained from this sample solution and reacted with a substrate solution at various pHs (3.0-9.0), which were adjusted by disodium hydrogenphosphate-citric acid buffer.

2.3 Purification of Chitinases

The crude extracellular enzyme was purified by ion exchange column chromatography on DEAE Sephadex A-50. This column was in advance equilibrated with the same buffer solution (pH 8.0) mentioned above at 4℃. After that, the column was washed with the same buffer solution at a flow rate of 0.35 $ml \cdot min^{-1}$. The bound proteins to the column were then eluted with 0-1.0 $mol \cdot dm^{-3}$ NaCl in the buffer.
Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the purity of chitinase and its molecular weight according to the method of Laemmli on 15 % acrylamide gel (Nishimura, 1997).

2.4 Chitin Hydrolysis Property of Purified Enzyme

The optimum pH of enzyme was measured after finishing the reaction of chitinases with glycol chitin solutions which were prepared at various pHs (3.0-9.0), at 37℃ for 20 h (Matsumoto, 2007). The optimum temperature of enzyme was also measured.
The maximum reaction rate and the Michaelis constant for the hydrolysis of glycol chitin by each chitinase were determined as explained in Subsection 3.3 (Yamane, 2003). After the chitinase reacted with a substrate solution of various concentrations, the chitinase activity was measured.
The hydrolysis products from the chitin oligomer, *N*-acetyl-chito-oligosaccharides ($(GlcNAc)_n$, n=4,5) by a chitinase Chi-3 were analyzed by a high performance liquid chromatography (Matsumoto, 2007).

3. Results and Discussion

3.1 Induction of Chitinase

A high chitinase activity was gained under some culture conditions. The best culture

medium was that containing chitin as a carbon source and no peptone as a nitrogen source. Under this culture condition, the highest chitinase activity was obtained at pH 3 and pH 4. The maximum chitinase activity was obtained at 24 h after the main cultivation was started.

3.2 Purification of Chitinases and Effects of pH and Temperature on Activity

As the result of purification of chitinase, the four fractions with a high chitinase activity were obtained as shown in *Figure 1* and these were named as Chi-1~4, respectively. Fraction number in the abscissa of *Figure 1* means the consecutive number of the sample solution collected by a fraction collector. The protein concentration of Chi-4 was too low to collect a necessary amount of Chi-4. Chi-3 was best purified to 1.75-fold among the chitinases. The molecular weights of Chi-1~3 were confirmed by SDS-PAGE to be 85, 30 and 55kDa for Chi-1~3, respectively.

The optimum pHs of Chi-1~3 were 8, 4 and 4, respectively as shown in *Figure 2*. The optimum temperatures of Chi-1~3 were 25, 37 and 37℃, respectively.

3.3 Determination of Maximum Reaction Rate and Michaelis Constant

One-substrate enzyme reaction proceeds generally according to the Michaelis-Menten mechanism as follows;

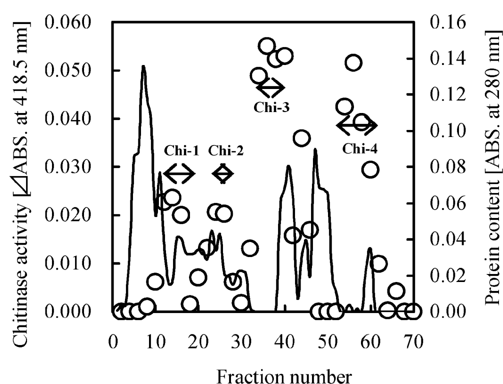
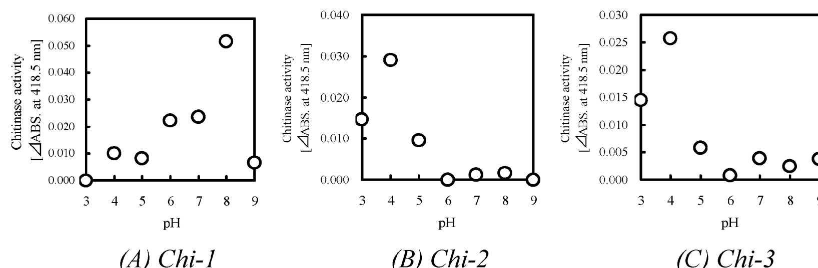


Figure 1: Ion-exchange columun chromatography of crude extracelluler enzyme on DEAE-Sephadex A-50 ○; Chitinase activity Curved line; Protein content



(A) Chi-1 *(B) Chi-2* *(C) Chi-3*

Figure 2: Effect of pH on activity of Chi-1~3

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P \quad (1)$$

Appling pseudo steady state approach to ES, the reaction rate V is obtained as follows;

$$V = -\frac{d[\mathrm{S}]}{dt} = \frac{k_{+2}[\mathrm{E}]_0[\mathrm{S}]}{K_\mathrm{m} + [\mathrm{S}]} = \frac{V_{\max}[\mathrm{S}]}{K_\mathrm{m} + [\mathrm{S}]} \quad (2)$$

Where $[E]_0$ is the total enzyme concentration, [S] is the substrate concentration, K_m is the Michaelis constant expressed as $K_m=(k_{-1}+k_{+2})/k_{+1}$, and V_{max} is the maximum reaction rate expressed as $V_{max}=k_{+2}[E]_0$. Then, equation (2) is rewritten to obtain equation (3).

$$\frac{1}{V} = \frac{1}{V_{\max}} + \frac{K_\mathrm{m}}{V_{\max}}\frac{1}{[\mathrm{S}]} \quad (3)$$

Plot of data according to equation (3) is called the Lineweaver-Burk plot.
The results for the reaction of Chi-3 with the substrate (glycol chitin) solution of various concentrations were shown in *Figure 3*. The Lineweaver-Burk plot based on *Figure 3* was done, and V_{max} and K_m were determined. Those for Chi-1 and Chi-2 were also determined by the same method. The results are summarized in *Table 1.*
The reactivity of Chi-3 was found to be the highest among the three chitinases.
Appling V_{max} and K_m determined to equation (2) in the case of Chi-3, the experimental results were well fitted to the theoretical curve as shown in *Figure 4*. Therefore, this enzyme reaction was found to obey the Michaelis-Menten reaction kinetics.

3.4 Kinetics of Hydrolysis Reaction of Chitin Oligomer
The results of hydrolysis reaction of N-acetyl-chito-oligosaccharides ($GlcNAc_5$ and $GlcNAc_4$) by Chi-3 were shown in *Figure 5*. In the hydrolysis reaction of pentamer, it was found from *Figure 5(A)* that trimer and dimer with the same molar quantity were produced. In the hydrolysis reaction of tetramer (*Figure 5(B)*), it was found that dimer of

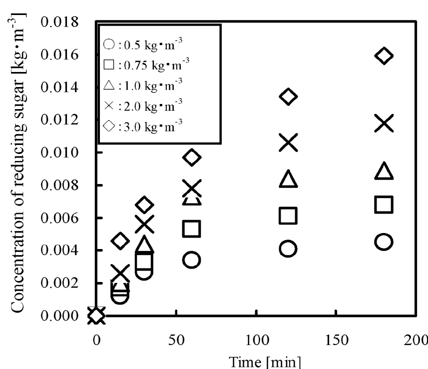


Figure 3: Time course of hydrolysis of glycol chitin by Chi-3

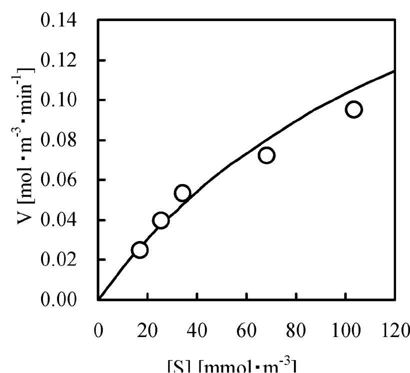


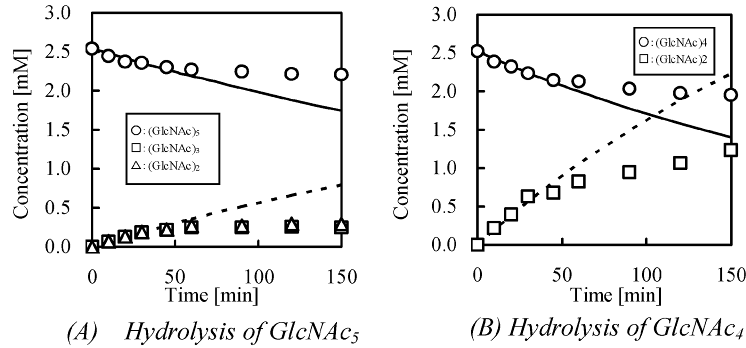
Figure 4: Relationship between V and [S] for hydrolysis of Chi-3

Table 1: V_{max} and K_m for glycol chitin hydrolysis

Chitinase	V_{max} [mol m^{-3} min^{-1}]	K_m [mol m^{-3}]
Chi-1	0.10	0.17
Chi-2	0.096	0.18
Chi-3	0.26	0.15

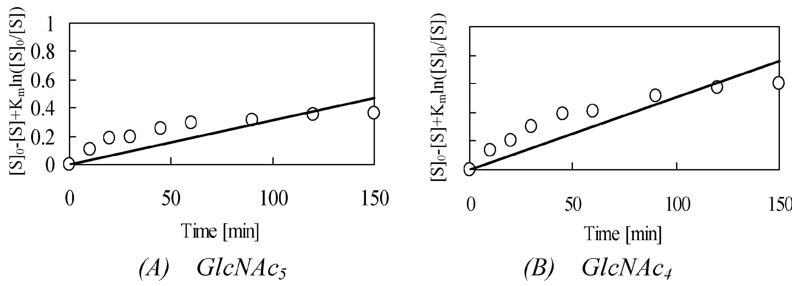
Table 2: V_{max} and K_m for oligomer hydrolysis by Chi-3

Oligomer	V_{max} [mol m^{-3} min^{-1}]	K_m [mol m^{-3}]
Pentamer	0.0032	0.15
Tetramer	0.0050	0.10



(A) Hydrolysis of $GlcNAc_5$ *(B) Hydrolysis of $GlcNAc_4$*

Figure 5: Time course of N-acetyl-chito-oligosaccharides by Chi-3



(A) $GlcNAc_5$ *(B) $GlcNAc_4$*

Figure 6: Plot of results for chitin oligomer hydrolysis according to equation (6)

two times the quantity of tetramer consumed was produced. The results lead to the following mechanism for the oligomer hydrolysis reaction.

$$(GlcNAc)_5 \rightarrow (GlcNAc)_3 + (GlcNAc)_2 \quad (4)$$

$$(GlcNAc)_4 \rightarrow 2(GlcNAc)_2 \quad (5)$$

As mentioned above, the reaction rate of the substance S is written by equation (2).
Equation (2) can be integrated under the initial condition of [S] = $[S]_0$ at t=0. As the result, equation (6) is obtained.

$$[S]_0 - [S] + K_m \ln \frac{[S]_0}{[S]} = V_{max} t \quad (6)$$

The experimental results for the hydrolysis reaction of the oligomers were arranged

according to equation (6), and were shown in *Figure 6*, from which the kinetic parameters for the hydrolysis reaction of pentamer and tetramer were obtained. The results are summarized in *Table 2*. The lines in *Figure 6* are the calculated ones using the parameters in *Table 2*. The parameters listed in *Table 2* were obtained as follows. The right-hand side of equation (6) can be calculated by assuming a value of K_m, and this is plotted against time t. By this procedure, the optimum value of K_m is obtained to explain the experimental data. As the results, the value of V_{max} is obtained from the slope of the straight line. The discrepancy of the experimental result from the calculated one in *Figure 6* may be due to taking the initial reaction rates into consideration in the analysis.
Here, the kinetic parameters for the hydrolysis reaction of glycol chitin and the oligomers are compared with each other. As shown in *Table 1*, the values of V_{max} and K_m for the hydrolysis of glycol chitin by Chi-3 are 0.26 $mol \cdot m^{-3} \cdot min^{-1}$ and 0.15 $mol \cdot m^{-3}$, respectively. On the other hand, the values of V_{max} and K_m for the hydrolysis of oligomer are 0.0032 $mol \cdot m^{-3} \cdot min^{-1}$ and 0.15 $mol \cdot m^{-3}$ for pentamer, and 0.0050 $mol \cdot m^{-3} \cdot min^{-1}$ and 0.10 $mol \cdot m^{-3}$ for tetramer. The maximum reaction rate V_{max} of glycol chitin is much bigger than those of the oligomer, meaning that the reactivity of glycol chitin is much higher than those of the oligomer. This is a reasonable result considering the saccharide chain length.

4. Conclusions

In this study, we extracted a crude protein containing chitinase from *Bacillus subtilis* and this was purified to obtain pure chitinases by ion exchange column chromatography. From the result of purification, three major fractions with a high chitinase activity were obtained and named as Chi-1~3, respectively. Chi-3 was purified to 1.75-fold; this was best purified among these. The molecular weights of Chi-1~3 were 85, 30, and 55kDa, respectively. The optimum pHs of Chi-1~3 were 8, 4, and 4, respectively. The optimum temperatures of Chi-1~3 were 25, 37, and 37℃, respectively.
For the hydrolysis reaction of glycol chitin by Chi-3, V_{max} was the largest and K_m was the smallest among the three chitinases. Chi-3 hydrolyzed *N*-acetyl-chito-oligosaccharides as follows ; $(GlcNAc)_5 \rightarrow (GlcNAc)_3 + (GlcNAc)_2$, and $(GlcNAc)_4 \rightarrow 2(GlcNAc)_2$. The Michaelis parameters for the hydrolysis reaction of the oligomer were obtained by applying the data directly to the Michaelis-Menten rate equation.

References

Chitin and chitosan research group, 1995, Handbook of Chitin and Chitosan, Gihodo, Tokyo, Japan.
Chitin and chitosan research group, 2004, Development and Application of Chitin and Chitosan, Gihodo, Tokyo, Japan.
Imoto T. and Yagishita K., 1971, A Simple Activity Measurement of Lysozyme, Agricultural and.Biological Chemistry, 35, 1154-1156.
Fukui T., 1991, Determination Method of Protein Quantity, Hirokawashoten, Tokyo, Japan.
Nishihira K., 1997, Bio-Experiment Illustrated 5, Shujunsha, Tokyo, Japan.
Matsumoto Y., 2007, Master Thesis of Chemical Engineering Department in Doshisha University, Kyoto, Japan.
Yamane T., 2003, Biochemical Reaction Engineering, Sangyotosyo, Tokyo, Japan.