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Kinetic Parameters of Endochitinase from *Bacillus licheniformis* MB-2

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ABSTRAK

Parameter Kinetika Endokitinase dari *Bacillus licheniformis* MB-2. *Bacillus licheniformis* MB-2 yang diisolasi dari air panas di Tompaso (Sulawesi Utara) menghasilkan 5 enzim kitinase ekstraselular. Salah satu enzim, Chi-67, telah dimurnikan dan dikarekterisasi. Chi-67 dapat menghidrolisis substrat kitin buatan maupun alami. Analisis kinetika aktivitas enzim terhadap berbagai substrat kitin telah dilakukan. Enzim dapat memotong 4-methylumbelliferyl *N', N'*-diacetylchitobioside {MUF(GlNAc)$_2$}, 4-methylumbelliferyl *N',N' N'*-triacetylchitotrioside {MUF(GlNAc)$_3$}, glikol kitin, dan koloidal kitin, tetapi tidak dapat melepaskan senyawa MUF dari 4-methylumbelliferyl *N*-acetyl-ß-D-glucosamine {MUF(GlNAc)}. Ini menunjukkan Chi-67 memiliki tipe pemotongan endo. Kurva kecepatan reaksi substrat menunjukkan pola profil Michaelis-Menten. Kecepatan reaksi maksimum (V_{max}) terhadap MUF(GlNAc)$_2$ identik dengan V_{max} terhadap MUF(GlNAc)$_3$ yaitu 0.02 µM.min^{-1} . V_{max} enzim terhadap koloidal kitin adalah 0.01 µM.h^{-1} atau lebih rendah 3 kali dari nilai V_{max} pada substrat glikol kitin (0.03 µM.h^{-1}). Nilai K_{cat} untuk koloidal kitin sebesar 8.01 h^{-1} atau hampir 2 kali lebih tinggi dari glikol kitin (4.32 h^{-1}) sementara K_{cat} untuk MUF(GlNAc)$_2$ adalah 3.65 min^{-1} atau 1,5 kali lebih tinggi dari K_{cat} MUF(GlNAc)$_3$ yang hanya 2.90 min^{-1} . Afinitas Chi-67 terhadap koloidal kitin, glikol kitin, MUF(GlNAc)$_2$, dan MUF(GlNAc)$_3$ yang ditunjukkan oleh nilai konstanta Michaelis (K_m) masing-masing 3.08 mg.ml^{-1}, 0.32 mg.ml^{-1}, 0.26 mM dan 0.1 mM.

Kata kunci: Bakteri, endokitinase, enzim, dan stabilitas panas

INTRODUCTION

Chitin, a β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) homopolymer, is one of the most abundant biopolymers in the biosphere, with an estimated annual production in nature is 10^{10} to 10^{11} tons (Gooday 1990). Chitin, for which specific structural features are source dependent, is the main structural component of most fungal cell walls and exoskeletons of arthropods and is found

in both terrestrial and marine habitats (Salmon & Hudson 1997). This polysaccharide serves as a carbon and energy source for a wide variety of organisms, and enzymes comprising various chitinolytic systems have been isolated and studied (Watanabe *et al.* 1997; Gal *et al.* 1998; Tanaka *et al.* 2001). Chitinases, separately or synergistically, hydrolyze chitin into small oligosaccharides by cleaving the β -1,4 linkages in the polymer backbone.

More than 80 families of glycosyl hydrolases have been defined on the basis of amino acid sequence homology (Davies a & Henrissat, 1995; Henrissat and Coutinho 2001). Chitinases are assigned to two families: 18 and 19. Family 18 includes chitinases from bacteria, plants, fungi, viruses, and animals and was recently further divided into three subfamilies, i.e., A, B, and C (Suzuki *et al.* 1999). Many chitinases have chitin-binding domains and catalytic domains that may be flanked or interspersed among one or more proline-threonine (PT) rich regions (Svitil & Kirchman 1998). The catalytic domains contain the catalytic residues, a glutamic acid and an aspartic acid, which have been identified through site-directed mutagenesis (Watanabe *et al.* 1993), based on information from crystal structures of two bacterial chitinases from *Serratia marcescens* (Perrakis *et al.* 1994; van Aalten *et al.* 2000). These two amino acids are thought to be involved in general acid catalysis in a retaining mechanism. Chitinases have been identified in microorganisms from a wide variety of thermal environments, ranging from a psychrophilic bacterium, *Arthrobacter* sp., growing at 4°C (Lonhienne *et al.* 2001) to moderately thermophilic bacteria (Takayanagi *et al.* 1991; Sakai *et al.* 1998), which are active up to a temperature range of 70 to 80°C.

One bacterium, *Bacillus licheniformis* MB-2, has been isolated from Tompaso hot spring water (Indonesia). MB-2 produced five extracellular chitinases continuously during 5 days when grown at 55 °C in medium containing 0.5% colloidal chitin. Their molecular masses were 78, 67, 61, 50 and 47 kDa which were tentatively named Chi-78, -67, -61, -50 and -47, respectively. The major chitinase produced by MB-2 i.e. the chitinase-67 kDa (Chi-67) was further studied. The enzyme molecule was most heat stable (90 °C, 60 min) amongst the 5 chitinases from MB-2 and present in highest concentrations (20 μg/ml) in the culture medium. The enzyme was purified from the extracellular culture by heat treatment, and successive chromatography steps including hydrophobic interaction, anion exchange and gel filtration chromatography. Although the enzyme has been analyzed for qualitatively regarding their stabilities toward various temperatures, pHs, denaturants and solvents (Toharisman *et al.* 2005), its kinetics has not been determined. Information of enzyme kinetics is very important in enzyme applications. This paper describes Chi-67 kinetics toward various substrates.

MATERIALS AND METHODS

Chemicals

Glycol chitosan, *N*-acetyl D-glucosamine, chitosan, chitin, 4-methylumbelliferyl *N'*-acetyl-ß-D-glucosaminide [MUF(GlNAc)], $MUF(GlNAc)_2$, $MUF(GlNAc)_3$ and Phenyl Sepharose CL-4B resin were purchased from Sigma-Aldrich Inc. DEAE Sephacel, Superdex 75, chromatography columns and Acta Purifier were obtained from Pharmacia LKB (Upsala, Sweden). Glycol chitin was prepared from glycol chitosan by the method of Trudel & Asselin (1989) with reacetylation using acetic anhydride. Regenerated chitin and colloidal chitin were prepared from chitosan by the method of Molano *et al.* (1977) and Sakai *et al.* (1998). All other chemicals were of p.a. quality.

Microorganism

Bacillus licheniformis MB-2 was isolated from Tompaso hot-water in North Sulawesi, Indonesia and purified on an agar plates containing colloidal chitin. The cells were cultured on LB medium supplemented with 0.3% colloidal chitin and incubated at 55 °C for 48 h on a rotary shaker at 200 rev. min^{-1}. Ten milliliters of preculture was used as an inoculum for five Erlenmeyer flasks, each containing 90 ml of medium with the following composition per liter: 0.5% colloidal chitin, 0.7% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% NaCl, 0.01% $MgSO_4.7H_2O$, 0.05% yeast extract and 1.5% agar (pH 7.0) and incubated at 55 °C on a rotary shaker at 180 $rev.min^{-1}$. The culture was centrifuged at 4 °C for 20 minutes at 10 000 g. The supernatant was collected for further purification.

Enzyme Purification

The supernatant (250 ml) was heated at 80 °C for 5 min and centrifuged as above to remove heat-labile proteins. The solution then was mixed with $(NH_4)_2SO_4$ to a final concentration of 1 M and the pH was adjusted to 8.0 with 1 M NaOH. The mixture was loaded to Phenyl Sepharose CL-4B column (1.6 x 15 cm) equilibrated with 20 mM Tris-Cl buffer pH 8.0 containing 1 M $(NH_4)_2SO_4$. After washing with 200 ml of the same buffer, the enzyme was eluted with a combination of a linear descending gradient between 1.0 and 0 M ammonium sulfate in 20 mM Tris-Cl, pH 8.0 and a linear gradient ascending gradient of Tween-20 between 0 and 1% (v/v) in 20 mM Tris-Cl, pH 8.0 at a flow rate of 10 ml h^{-1} (2 ml per tube).

Fractions with chitinase activity were pooled, concentrated with Amicon Ultrafiltration (NMWL 10kDa, Millipore) and subjected to DEAE-Sephacel column (1.6 x 15 cm) equilibrated with 10 mM Tris-Cl, pH 8.0. Bound proteins were eluted using a combined linear ascending gradient between 0 and 0.5 M NaCl in 10 mM Tris-Cl (A-B), pH 8.0 and 0 and 1% (v/v) Tween-20 in 10 mM Tris-Cl, pH 8.0. Active fractions were pooled and concentrated as above, applied to HiLoad Superdex 75 column (1.6 x 60 cm) equilibrated with 10 mM Tris-Cl, pH 8.0 at a flow rate of 0.1 ml min^{-1} (1 ml

per tube), and eluted with the same buffer. Active fractions were pooled, concentrated and used for further study. All purification steps were performed at ambient temperature except for gel filtration chromatography (4 °C).

Enzyme Assay

For isolation and substrate specificity experiments chitinase activity was measured by a colourimetric method using colloidal chitin as substrate (Yanai *et al.* 1992. The enzyme solution (100 ml) was mixed with 300 ml of 0.3% (w/v) colloidal chitin dissolved in McIlvaine buffer (pH 6.0) and incubated at 60 °C for 30 min. The control was prepared by adding inactivated enzyme (cooling for 10 min) after incubation. The solution was then centrifuged at 10 000 g, 4 °C for 5 min. The amount of reducing sugars produced was determined at 420 nm with a modified Schales method (Ueda *et al.* 1996). One unit of chitinase activity was defined as the amount of enzyme that liberated 1 mmol of reducing sugar as *N*-acetylglucosamine (GlcNAc) equivalent per min.

For other studies, chitinase activity was measured using a fluorimetric assay each of following substrates: 1 mM MUF(GlNAc), 250 µM MUF$(GlNAc)_2$, and 250 µM MUF$(GlNAc)_3$ (Spindler 1997). The enzyme (20 µl) was mixed with 50 µl substrate and 30 µl McIlvaine buffer (pH 6.0), and incubated for 30 min at 60 °C. The reaction was stopped with 100 µl of 1 M glycine-NaOH, pH 11.0. The amount of 4-methylumbelliferone released from the substrate was measured at an excitation wavelength of 360 nm and emission wavelength of 460 nm with a Cytoflour Multi-Well Plate Reader series 4000 (PerSeptive Biosystems).

Enzyme Kinetics

K_m and V_{max} for colloidal chitin and glycol chitin were determined for the purified enzyme using the colorimetric assay described above, whereas for MUF$(GlNAc)_2$ and MUF$(GlNAc)_3$ the fluorimetric assay was used. The K_m and V_{max} were calculated from direct linear plots (Lineweaver Burk) of the data.

Protein Determination

Protein was determined by measuring the absorbance at 280 nm during chromatographic separation. Protein was also determined by the method of Bradford (1976) with bovine serum albumin fraction V as a standard.

RESULTS

Chi-67 was purified by successive hydrophobic interaction, anion exchange, and gel filtration chromatographies. The enzyme was a monomer with an apparent molecular weight of 67 kDa (Table 1 and Figure 2A). Chi-67 could hydrolyze colloidal chitin, glycol chitin, chitosan and glycol chitosan (Table 2).

Kinetic parameters of Chi-67 was studied using natural (colloidal chitin and glycol chitin) and artificial substrates

($MUF(GlcNAc)_2$ and $MUF(GlcNAc)_3$). A linear relationship between the rate of $MUF(GlcNAc)_2$ hydrolysis and incubation time was shown on Figure 2 and the effect of enzyme concentrations on the rate of MUF release was presented in Figure 2B. Lineweaver-Burk plot of Chi-67 for colloidal chitin, glycol chitin, $MUF(GlcNAc)_2$, and $MUF(GlcNAc)_3$ and kinetic parameters of the enzyme on natural and artificial substrate were shown on Figure 3 and Table 3, respectively.

DISCUSSIONS

Bacillus licheniformis MB-2 is one of the isolates isolated from Tompaso hot spring (North Sulawesi, Indonesia) forming clear zone when it was grown on solid medium containing colloidal chitin as a source of carbon. MB-2 produced five extracellularly chitinases continuously during 5 days when grown at 55 °C in medium containing 0.5% colloidal chitin. Their molecular masses were 78, 67, 61, 50 and 47 kDa which were tentatively named Chi-78, -67, -61, -50 and -47, respectively (Table 1; Figure 1). The major chitinase produced by MB-2 i.e. the chitinase-67 kDa (Chi-67) was further studied. The enzyme molecule was the most heat stable amongst the 5 chitinases from MB-2 and present in highest concentrations in the culture medium. The enzyme was purified from the extracellular culture by heat treatment, and successive chromatography steps.

Multiform chitinases in the range of 30-81 kDa produced by bacteria have been reported previously. In the case of *Bacillus* MH-1, three isoform chitinases corresponding to 71, 62 and 53 kDa

Table 1. Purification steps of Chi-67 produced by *Bacillus licheniformis* MB-2

Purification step	Total protein (mg)	Total activity (U)	Spec. Activity (U/mg)	Yield (%)	Purification fold
Cell free supernatant	21.50	3 750	174	100.00	1.00
Heat treatment (80 °C, 5 min)	12.80	3 050	238	81.34	1.37
Phenyl Sepharose CL-4B	0.86	672	782	17.94	4.49
DEAE Sephacel	0.44	560	1 274	14.94	7.30
Superdex 75	0.05	267	2 673	3.56	15.33

Table 2. Substrate specificity of the chitinase

Substrate	Relative Activity (%)
Colloidal chitin	100.00
Glycol chitin	85.41
Regenerated chitin	33.72
Fine powder of chitin	1.14
Chitosan	67.44

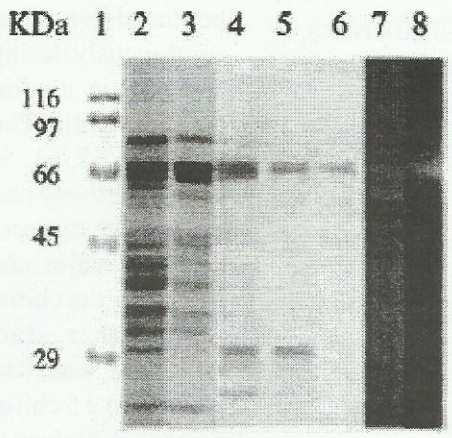

Figure 1. SDS-PAGE of peak fractions from different purification steps. Lane 1, molecular weight markers; lane 2, crude extract enzymes; lane 3, proteins derived from heating 80 °C for 5 min; lane 4, proteins from hydrophobic interaction chromatography; lane 5, proteins from anion exchanger; lane 6, protein from gel filtration; lane 7, zymogram analysis with 0.1% glycol chitin of band from lane 2; lane 8, zymogram analysis with MUF(GlNAc)$_3$ of band from lane 6

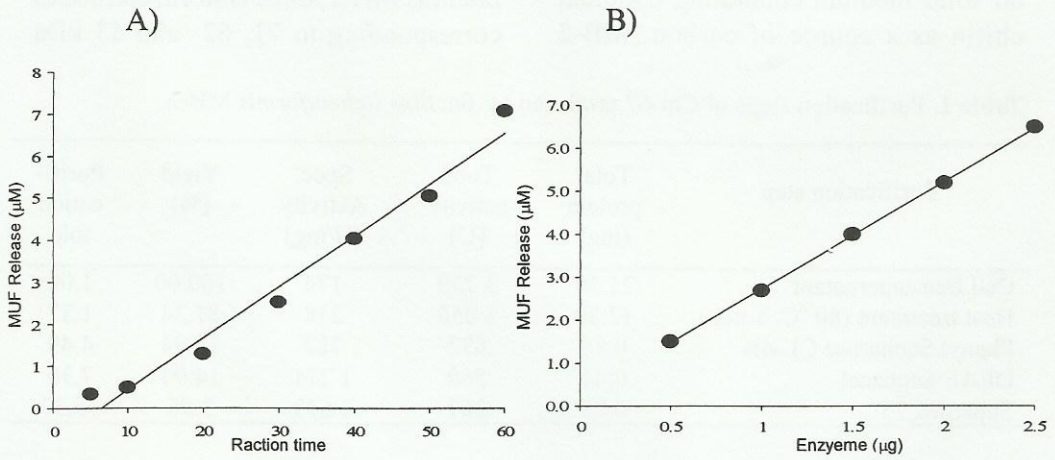

Figure 2. Chitinase activities on flourogenic substrates as a function of incubation time. The amount of protein was 1-2 µg and MUF(GlcNAc)$_2$ concentrations was 200 µg/ml (A); Chitinase activities on flourogenic substrates as a function of enzyme. Chi-67 was reacted with MUF(GlcNAc)$_2$ for 30 min incubation time. MUF(GlcNAc)$_2$ concentrations was 200 µg/ml (B)

Table 3. Kinetic parameters of Chi-67 for natural and artificial chitins

Substrate	Kinetic Parameters			
	V_{max}	K_m	K_{cat}	K_{cat}/K_m
Colloidal Chitin	0.01 $\mu M.h^{-1}$	3.08 mg ml^{-1}	8.01 h^{-1}	2.60 $ml.mg^{-1}.h^{-1}$
Glycol Chitin	0.03 $\mu M.h^{-1}$	0.32 mg ml^{-1}	4.32 h^{-1}	13.50 $ml.mg^{-1}.h^{-1}$
$MUF(GlcNAc)_2$	0.02 $\mu M.min^{-1}$	0.26 mM	3.65 min^{-1}	14.04 $(min.mM)^{-1}$
$MUF(GlcNAc)_3$	0.02 $\mu M.min^{-1}$	0.10 mM	2.90 min^{-1}	29.00 $(min.mM)^{-1}$

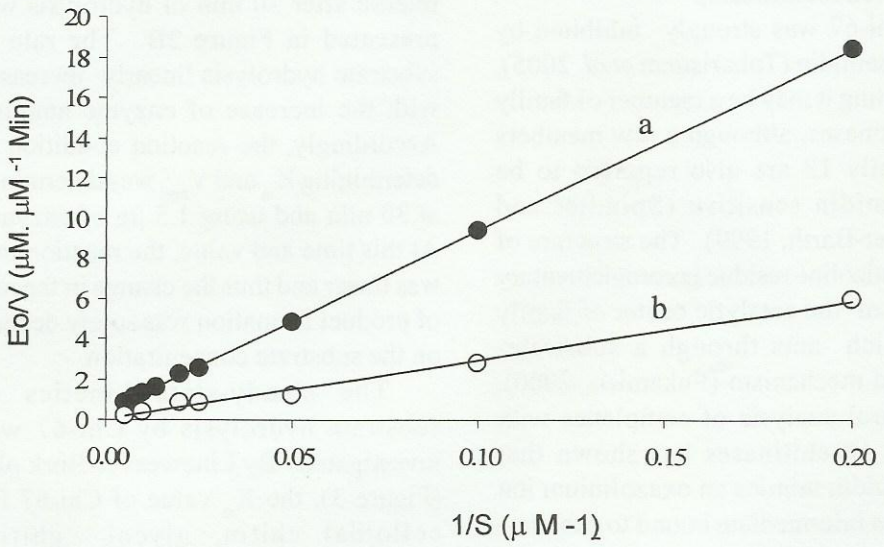

Figure 3. Lineweaver-Burk plots of Chi-67 using $MUF(GlcNAc)_2$ (a) and $MUF(GlcNAc)_3$ (b) as substrates

were found (Sakai *et al.* 1998). Three strains of *Vibrio algynolyticus* have two chitinases with different molecular massas (Murao *et al.* 1992; Ohishi *et al.* 1997). The bacteria produced several chitinases, probably to hydrolyze the diversity of chitins found in nature. Chitin can vary by the arrangement of *N*-acetylglucosamine strands, degree of deacetylation, and presence of cross-linked structural components such as protein and glucans (Gooday 1990).

Chi-67 hydrolyzes colloidal chitin, glycol chitin, chitosan and glycol chitosan (Table 2). This suggests that the chitin binding domain posseses wide range of binding capability for various chitinous substrates which is similar to chitinases from *B. circulans* WL-12 (Watanabe *et al.* 1993), *Bacillus* strain

MH-1 (Sakai *et al.* 1998), and *S. olivaceoviridis* (Blaak *et al.* 1993). This is in agreement with fact that the enzyme activity was reduced in the presence of DTT, since the typical chitin binding domain has a consensus sequence of $CX_{11}CX_5CX_9CX_{12}CX_7C$ and the cysteins are connected by S-S- bridges (Hayashi *et al.* 1995). The enzyme degrades glycol chitin, $MUF(GlcNAc)_2$ and $MUF(GlcNAc)_3$ but not MUF (GlcNAc), indicating an endo-spliting mode of action (endochitinase).

Chi-67 was strongly inhibited by of allosamidin (Toharisman *et al.* 2005), suggesting it may be a member of family 18 chitinases, although a few members of family 19 are also reported to be allosamidin sensitive (Spindler and Spindler-Barth, 1999). The structure of allosamizoline residue is complementary to that of the catalytic center of family 18 which acts through a substrate-assisted mechanism (Fukamizo, 2000). Structural analysis of complexes with family 18 chitinases has shown that allosamidin mimics an oxazolinium ion reaction intermediate bound to subsite –3 through –1. During normal catalysis, this oxazolinium ion intermediate is formed by nucleophilic attack of the N-acetyl group of the –1 sugar on the anomeric carbon, which occur concomitantly with protonation and breakage of the scissile glycosidic bond by the catalytic acid (van Aalten *et al.* 2002).

Kinetics parameters of Chi-67 was studied with the purified enzyme. Kinetic experiments with natural substrates were performed using the reducing sugar assay, whereas for synthetic substrates carrying the methylumbelliferon (MUF) group were performed using the flourometric assay. The rate of product formation for artificial chitin substrates at the optimum temperature and enzyme concentration was analyzed as a function of time. Figure 2 shows a linear relationship between the rate of $MUF(GlcNAc)_2$ hydrolysis and incubation time. Based on this result, the effect of enzyme concentrations on the rate of MUF release after 30 min of hydrolysis was presented in Figure 2B. The rate of substrate hydrolysis linearly increased with the increase of enzyme amount. Accordingly, the reaction condition in determining K_m and V_{max} was determined at 30 min and using 1.5 µg of enzyme. At this time and value, the reaction rate was linear and thus the change in the rate of product formation was solely depend on the substrate concentration.

The steady-state kinetics of substrate hydrolysis by Chi-67 was investigated. By Lineweaver-Burk plot (Figure 3), the K_m value of Chi-67 for colloidal chitin, glycol chitin, $MUF(GlcNAc)_2$, and $MUF(GlcNAc)_3$ were estimated to be 3.08 mg ml^{-1}, 0.32 mg ml^{-1}, 0.26 mM and 0.10 mM, respectively (Table 3). The maximum velocity (V_{max}) obtained with $MUF(GlcNAc)_2$ was identical to the V_{max} value observed for $MUF(GlcNAc)_3$. The enzyme hydrolyzed colloidal chitin with a 3-fold lower V_{max} value compared to glycol chitin. The K_{cat} value for colloidal chitin was almost 2 times higher than that of glycol chitin, whereas

K_{cat} for MUF $(GlcNAc)_2$ was 1.5 times higher than that of MUF$(GlcNAc)_3$.

The determination of kinetic constants with 4 substrates showed that the K_m of Chi-67 decreased by 1 order of magnitude when colloidal chitin was replaced with glycol chitin, but this was also accompanied by a 2-fold decrease in K_{cat}. The K_m and K_{cat} of MUF $(GlcNAc)_2$ were higher than those of MUF$(GlcNAc)_3$. Brurberg *et al.* (1996) reported that the K_m of an endochitinase form *Serratia marcescens* BJL2000 using the same substrate was 34.1 μM, whereas for endochitinase from *Clostridium paraputrificum* was only 6.3μM (Morimoto *et al.* 1997). K_m and V_{max} of exochitinase from *Streptomyces thermoviolaceus* OPC-520 were 425.7μM and 24.8 μmol min^{-1} mg of $protein^{-1}$, respectively (Tsujibo *et al.* 1998).

CONCLUSION

Bacillus licheniformis MB-2 which was isolated from Tompaso hot spring water produced several extracellular chitinases when the cell was grown on medium containing colloidal chitin. One of the enzymes, Chi-67, has been purified and further characterized. Chi-67 hydrolyzed soluble substrates much more efficiently than insoluble substrates. Among the chitin-related substrates tested, colloidal chitin was the best substrate for the enzyme.

The catalytic eficiency (K_{cat}/K_m) and the K_m of the enzyme on glycol chitin were 13.50 $ml.mg^{-1}.h^{-1}$ and 0.32 mg ml^{-1}, respectively, meanwhile on colloidal chitin were 2.60 $ml.mg^{-1}.h^{-1}$ and 3.08 mg ml^{-1}, respectively. The kinetic constants decreased by 1 order of magnitude when colloidal chitin (3.08 mg ml^{-1}) was re-placed with glycol chitin (0.32 mg ml^{-1}). The K_m and K_{cat} of MUF$(GlcNAc)_2$ were 0.26 mM and 3.65 min^{-1}, respectively. These values were higher than those of MUF(GlcNAc).

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