Kinetics of Chitinase from Yam, *Dioscorea opposita* THUNB

Daizo KOGA,* Tsuyoshi TSUKAMOTO, Nobuyuki SUESHIGE,
Toshihiko UTSUMI and Akio IDE

Laboratory of Biochemistry, Faculty of Agriculture, Yamaguchi University,
Yamaguchi 753, Japan

Received March 20, 1989

Kinetic analysis was done on chitinase E3 (EC 3.2.1.14) from yam, *Dioscorea opposita* THUNB, using both series of *N*-acetylchitooligosaccharides (GlcNAc_n, *n* = 2 to 6) and *p*-nitrophenyl *N*-acetylchitooligosaccharides (pNp-GlcNAc_n, *n* = 1 to 5) as substrates. The enzyme cleaved GlcNAc₃ to GlcNAc plus GlcNAc₂, GlcNAc₄ to two molecules of GlcNAc₂, GlcNAc₅ to GlcNAc₂ plus GlcNAc₃, and GlcNAc₆ by three ways to GlcNAc plus GlcNAc₅ (32%), GlcNAc₂ plus GlcNAc₄ (42%) and two molecules of GlcNAc₃ (26%). The speed of the reaction was observed in the following order, GlcNAc₄ > GlcNAc₅ > GlcNAc₆ > GlcNAc₃. Stronger substrate inhibition was observed in the longer chain substrates. The reactions of pNp-GlcNAc_n (*n* = 1 to 5) were similar to those of GlcNAc_n (*n* = 2 to 6), respectively. The cleavage sites of pNp-GlcNAc₂ and pNp-GlcNAc₃ were the second β-1,4-linkages from the reducing end side, and the main cleavage sites of pNp-GlcNAc₄ and pNp-GlcNAc₅ were the third linkages. *p*-Nitrophenol was not released from all *p*-nitrophenyl derivatives. The speed of the reaction was observed in the following order, pNp-GlcNAc₄ > pNp-GlcNAc₅ > pNp-GlcNAc₃ > pNp-GlcNAc₂. Neither GlcNAc₂ nor pNp-GlcNAc was cleaved. These results suggest that yam chitinase E3 acts in a random fashion.

Yam chitinase is good for releasing protoplasts from basidiomycetes,¹⁾ and is resistant to allosamidin, which is a specific inhibitor of insect chitinase.²⁾ These characteristics are different from insect chitinases, and the differences seem to be related to their roles in physiology. In insects, two types of chitinolytic enzymes, chitinase and β-*N*-acetylglucosaminidase, act cooperatively in ecdysis,³⁾ while the physiological role of plant chitinase is self-defense against chitin-containing invaders such as plant pathogens and pests.^{4,5)} It is easily expected that the reaction pattern and substrate specificity of yam chitinase are different from those of other chitinases.

In this study, we did a kinetic analysis on chitinase E3 from yam using both series of *N*-acetylchitooligosaccharides and pNp-*N*-acetylchitooligosaccharides, and discussed the cleavage patterns and sites.

Materials and Methods

Materials. *N*-Acetylchitooligosaccharides (GlcNAc_n, *n* = 1 to 6) were prepared from crab chitin (a generous gift from Katakurachikkari Co., Ltd., Tokyo) by the method of Raftery *et al.*⁶⁾ *p*-Nitrophenyl *N*-acetylchitooligosaccharides (pNp-GlcNAc_n, *n* = 1 to 5) were generous gifts from Yaizu Suisankagaku Industry Co., Ltd., Shizuoka, Japan. Other reagents were all of analytical grade.

Enzyme. Chitinase E3 was purified from yam, *Dioscorea opposita* THUNB, by the method of Tsukamoto *et al.*⁷⁾ and lyophilized for storage. The appropriate concentration of enzyme was prepared by dissolving the lyophilized enzyme in distilled water using a molar extinction coefficient of 47,000.⁷⁾

Enzyme assay. Chitinase activity was measured by HPLC. Ten microliters of the enzyme solution (final concentration, 40 to 630 nM) was added to 100 μl of GlcNAc_n (*n* = 2 to 6) (final concentration, 0.05 to 0.2 mM) or pNp-GlcNAc_n (*n* = 1 to 5) (final concentration, 0.1 mM) dissolved in Britton-Robinson buffer,⁸⁾ pH 3.8, and in-

* To whom correspondence and reprint requests should be addressed.

Abbreviations: GlcNAc, 2-acetamido-2-deoxy-β-D-glucopyranoside; GlcNAc_n, *N*-acetylchitooligosaccharides; HPLC, high performance liquid chromatography; pNp-GlcNAc_n, *p*-nitrophenyl *N*-acetylchitooligosaccharides.

cubated at 25°C. After an appropriate time (10 to 90 min), the reaction was stopped by boiling for 5 min. A 10- μ l portion of the reaction mixture was injected into a Tosoh TSK Gel G2000 PW column (0.75 $\times$ 60 cm) with Tosoh TSK PWL Guard column (0.75 $\times$ 7.5 cm) as a first column, and eluted with distilled water or 10% ethanol, or injected into a Shimadzu PNH₂-10 column (0.45 $\times$ 25 cm) and eluted with 85% ethanol. The *N*-acetylchitooligosaccharides eluted were monitored at 210 nm.⁹⁾

Kinetic analysis. Kinetic parameters were calculated by the non-linear optimization method described elsewhere¹⁰⁾ with a NEC ACOS-850 computer (Yamaguchi University).

Results

Substrate specificity and cleavage pattern

To investigate the substrate specificity and cleavage pattern, the enzymatic reactions were done with GlcNAc_{*n*} (*n*=2 to 6) as the substrates. The results are shown in Table I. Yam chitinase E3 cleaved GlcNAc₃ to GlcNAc plus GlcNAc₂, GlcNAc₄ to two molecules of GlcNAc₂, GlcNAc₅ to GlcNAc₂ plus GlcNAc₃, and GlcNAc₆ to all species of GlcNAc_{*n*} (*n*=1 to 5). GlcNAc₂ was not cleaved. The release of GlcNAc was observed in the early reactions of GlcNAc₃ and GlcNAc₆. Products with longer chains than the substrate

were not observed in any case. The speed of the reaction was observed in the following order, GlcNAc₄ > GlcNAc₅ > GlcNAc₆ > GlcNAc₃.

To locate the cleavage site, pNp-GlcNAc_{*n*} (*n*=1 to 5) were used as the substrates. The results are shown in Table II. The speed of the reaction was observed in the following order, pNp-GlcNAc₄ > pNp-GlcNAc₅ > pNp-GlcNAc₃ > pNp-GlcNAc₂. pNp-GlcNAc was not cleaved. An endo-type cleavage pattern was observed except for pNp-GlcNAc₂ which was cleaved very slowly to GlcNAc plus pNp-GlcNAc. Release of *p*-nitrophenol was not observed in all cases. The cleavage sites of pNp-GlcNAc₂ and pNp-GlcNAc₃ were the second β -1,4-linkages from the reducing end, and the main cleavage sites of pNp-GlcNAc₄ and pNp-GlcNAc₅ were the third linkages.

Kinetic analysis

We have conducted a detailed kinetic analysis of the hydrolysis of a series of *N*-acetylchitooligosaccharides ranging in chain length from 2 to 6. The results are represented as double reciprocal plots in Figs. 1 and 2. Substrate inhibition was observed in the re-

Table I. SUBSTRATE SPECIFICITY OF YAM CHITINASE E3

Yam chitinase E3 (40 nm) was incubated with 0.1 mM *N*-acetylchitooligosaccharide in Britton-Robinson buffer, pH 3.8, at 25°C. After an appropriate time, the reaction mixture was boiled for 5 min, and a 10- μ l portion was analyzed by HPLC.

Substrate	Reaction	Initial velocity <i>v</i> ₀ (μ M/min)	Cleavage pattern
GlcNAc ₂ (II)		0	and/or
GlcNAc ₃ (III)	III $\rightarrow$ I + II	0.005 $\pm$ 0.003	G [↓] G [↓] G
GlcNAc ₄ (IV)	IV $\rightarrow$ 2II	1.782 $\pm$ 0.067	G-G [↓] G-G and/or
GlcNAc ₅ (V)	V $\rightarrow$ II + III	1.005 $\pm$ 0.066	G-G [↓] G [↓] G-G
GlcNAc ₆ (VI)	VI $\rightarrow$	0.877 $\pm$ 0.078 (100%)	
	$\rightarrow$ I + V	0.281 $\pm$ 0.011 (32%)	and/or G [↓] G-G-G-G [↓] G
	$\rightarrow$ II + IV	0.371 $\pm$ 0.055 (42%)	and/or G-G [↓] G-G [↓] G-G
	$\rightarrow$ 2III	0.225 $\pm$ 0.017 (26%)	G-G-G [↓] G-G-G

Mean values $\pm$ SE, *n* = 3.

I, GlcNAc.

Table II. SUBSTRATE SPECIFICITY OF YAM CHITINASE E3

The reactions were done as described in Table I, but the substrates were pNp-*N*-acetylchito oligo-saccharides.

Substrate	Reaction	Initial velocity v_0 ($\mu\text{M}/\text{min}$)	Cleavage pattern
pNp-GlcNAc (P-I)		0	
pNp-GlcNAc ₂ (P-II)	P-II $\rightarrow$ I + P-I	0.008 ± 0.002	G $\downarrow$ -G-P
pNp-GlcNAc ₃ (P-III)	P-III $\rightarrow$ II + P-I	0.192 ± 0.027	G-G $\downarrow$ -G-P
pNp-GlcNAc ₄ (P-IV)	P-IV $\rightarrow$	1.499 ± 0.057	
	$\rightarrow$ II + P-II	1.172 ± 0.046	G-G $\downarrow$ -G-G-P
	$\rightarrow$ III + P-I	0.327 ± 0.062	G-G-G $\downarrow$ -G-P
pNp-GlcNAc ₅ (P-V)	P-V $\rightarrow$	1.093 ± 0.211	
	$\rightarrow$ II + P-III	0.277 ± 0.064	G-G $\downarrow$ -G-G-G-P
	$\rightarrow$ III + P-II	0.619 ± 0.156	G-G-G $\downarrow$ -G-G-P
	$\rightarrow$ IV + P-I	0.157 ± 0.036	G-G-G-G $\downarrow$ -G-P

Mean values $\pm$ SE, $n=3$.

Fig. 1. Double Reciprocal Plots for Enzymatic Reactions of Yam Chitinase E3.

Ten microliters of the enzyme solution (final concentration, 40 nM for GlcNAc₄, GlcNAc₅ and GlcNAc₆ or 630 nM for GlcNAc₃) was added to 100 μl of *N*-acetylchito oligosaccharides (final concentrations, 0.05 to 0.2 mM) dissolved in Britton-Robinson buffer, pH 3.8, and incubated at 25°C. After an appropriate time, the reaction mixture was boiled for 5 min. A 10- μl portion of the reaction mixture was analyzed by HPLC. See Materials and Methods for details. The substrates: Panel A, GlcNAc₃ (III); Panel B, GlcNAc₄ (IV) and GlcNAc₅ (V); and Panel C, GlcNAc₆ (VI). Mean values $\pm$ SE, $n=3$.

Fig. 2. Double Reciprocal Plots for Enzymatic Reactions of Yam Chitinase E3.

The enzymatic reactions and HPLC analysis were done as described in Fig. 1, but the final concentrations of the enzyme were 100 nM for GlcNAc₄ and GlcNAc₅ and 160 nM for GlcNAc₆. The substrates: Panel A, GlcNAc₄ (IV); Panel B, GlcNAc₅ (V); and Panel C, GlcNAc₆ (VI). Mean values $\pm$ SE, $n=3$ except for GlcNAc₄ ($n=1$).

Table III. KINETIC PARAMETERS FOR YAM CHITINASE E3

The reactions were done as described in Table I. The kinetic parameters were calculated by the non-linear method.¹⁰⁾

Substrate	Concentration		k_{cat} (sec ⁻¹)	Km (mM)	k_{cat}/Km (mM ⁻¹ sec ⁻¹)
	Enz (nM)	Sub (μ M)			
GlcNAc ₃	630	50 ~ 200	0.044 ± 0.022	0.880 ± 0.527	0.050
	40	50 ~ 200	0.913 ± 0.049	0.025 ± 0.006	36.2
	100	50 ~ 200	0.990 ± 0.099	0.020 ± 0.010	49.0
GlcNAc ₅	40	50, 67	0.513 ± 0.115	0.014 ± 0.017	36.6
	100	50 ~ 100	0.540 ± 0.040	0.017 ± 0.007	32.4
GlcNAc ₆	160	50 ~ 100	0.403 ± 0.072	0.010 ± 0.014	38.7

Mean values $\pm$ SE, $n=4 \sim 12$.

actions of the longer chain substrates at the low concentration of the enzyme (40 nM). However, it decreased or disappeared at the high concentration of the enzyme (100 or 160 nM). The kinetic parameters calculated by the non-linear optimization method are shown in Table III. The Km decreased as the substrate chain length increased, while the k_{cat} was not

dependent on the chain length. A high k_{cat} was observed in GlcNAc₄. The relationships between the overall reactions (k_{cat}/Km) and the chain lengths reflected the initial velocities indicated in Table I.

Discussion

From the results of enzymatic reactions, it was found that yam chitinase E3 cleaves both GlcNAc_n ($n=3$ to 6) and pNp-GlcNAc_n ($n=2$ to 5). The reaction products suggest that yam chitinase is a random-type hydrolytic enzyme. GlcNAc₄ was cleaved in a specific way to two molecules of GlcNAc₂. However, there are some cleavage ways in the reactions of other substrates as indicated in Table I. From the reaction products of GlcNAc₆, it may be easily seen that the substrate is cleaved in three ways, namely to GlcNAc plus GlcNAc₅, GlcNAc₂ plus GlcNAc₄ and two molecules of GlcNAc₃. However, there are two further possible ways for the cleavage reaction producing two hetero-products such as GlcNAc_n and GlcNAc_m ($n \neq m$). This should be also considered in the cases of GlcNAc₃ and GlcNAc₅. The results of the reactions of pNp-GlcNAc_n ($n=1$ to 5) may be useful to find the cleavage site of *N*-acetylchitooligosaccharides. As shown in Table II, the cleavage of pNp-GlcNAc₂ occurred at a specific site, the second linkage from the reducing end. Therefore, it may be deduced that the cleavage site of GlcNAc₃ is the second linkage from the reducing end. Comparing with the reaction pattern of pNp-GlcNAc₄, it may be deduced that the main cleavage site of GlcNAc₅ is the third linkage from the reducing end. However, the difference in conformation between *p*-nitrophenyl and GlcNAc moieties should be taken into consideration. Since *p*-nitrophenol was not released in all cases, it is thought that the first β -1,4-linkage from the *p*-nitrophenyl moiety at the reducing end would not be cleaved. It may be deduced that the *p*-nitrophenyl moiety is not bound to the binding site adjacent to the reactive site, or that at least one GlcNAc moiety is required for binding to the binding site at the reducing end. In the case of GlcNAc₆, *p*-nitrophenol or GlcNAc was not released from pNp-GlcNAc₅ although GlcNAc was released in the reaction of GlcNAc₆. It may be deduced that the GlcNAc released from GlcNAc₆ is the moiety at the re-

ducing end, and that the corresponding *p*-nitrophenyl moiety of pNp-GlcNAc₅ is not cleaved for its steric hindrance. Two cleavage ways of GlcNAc₆ to GlcNAc₂ plus GlcNAc₄ may be possible as indicated in Table I, because pNp-GlcNAc₅ was cleaved in two ways to GlcNAc₂ plus pNp-GlcNAc₃ and GlcNAc₄ plus pNp-GlcNAc with the ratio of about 7:4. However, it is still difficult to decide which linkage is preferential, because the effect of *p*-nitrophenyl moiety on the binding is unknown. Comparing the cleaving rate of GlcNAc₄ with that of pNp-GlcNAc₃, it may be deduced that the *p*-nitrophenyl moiety strongly inhibited the reaction. It is suggested that the conformation is very crucial for the substrate to be recognized and bound to the binding sites adjacent to the reactive site. Therefore, some care is needed to decide the cleavage sites of *N*-acetylchitooligosaccharides from the results of the reactions of *p*-nitrophenyl *N*-acetylchitooligosaccharides. The cleavage sites estimated as mentioned above are shown in Tables I and II, and the main cleavage sites are indicated by big arrows.

With respect to the substrate specificity, a faster reaction was observed in GlcNAc₄ rather than GlcNAc₅ or GlcNAc₆ (Table I). The reaction velocity may be due to the probability of formation of the productive enzyme-substrate complex or to the degree of the affinity. However, as the substrate chain length increased from 3 to 6, the *K_m* decreased and the affinity increased. (Table III) That is, although the binding is strong, the reaction is slow. It may be deduced that the non-productive enzyme-substrate complexes are more frequently formed in the cases of GlcNAc₅ and GlcNAc₆ than GlcNAc₄.

Comparing with insect chitinases from *Manduca sexta*¹¹⁾ and *Bombyx mori* (Koga *et al.*, unpublished data), the insect chitinases preferentially cleave GlcNAc₅ and GlcNAc₆ rather than GlcNAc₄. The activities of the insect chitinases are about 4 times higher than that of yam chitinase toward GlcNAc₅, and 2 or 3 times higher toward glycol chitin.⁷⁾ That

is, the insect chitinases have higher activities than yam chitinase. However, the difference in the ratio between the long and short substrates seems to be due to the reactivity toward glycol chitin which is a chitin partially modified with ethylene glycol groups. The insect chitinases are largely affected by the degree of ethylene glycol groups, compared with yam chitinase. There is also a difference in the cleavage pattern of GlcNAc₆ between yam chitinase and the insect enzymes. The insect chitinases cleave GlcNAc₆ by only two ways to GlcNAc₂ plus GlcNAc₄ and two molecules of GlcNAc₃, while yam chitinase does by three ways to GlcNAc plus GlcNAc₅ in addition to the above two ways. (Table I) These differences in the enzymatic reaction between yam chitinase and the insect chitinases seem to be related to those in molecular size. The molecular weight of yam chitinase is 33.5 kDa,⁷⁾ while those of the insect chitinases are about 50 to 85 kDa.^{11,12)} The different characteristics in enzymatic reaction may be due to their physiological roles. Yam chitinase acts in self-defense against plant pathogens (Koga *et al.*, unpublished data) like other plant chitinases,^{4,5)} while insect chitinase acts in ecdysis.³⁾ Therefore, plant chitinase may have a wide

substrate specificity, while insect chitinase may have a strict one with high activity.

References

- 1) D. Koga, N. Sueshige, K. Orikono, T. Utsumi and A. Ide, *Agric. Biol. Chem.*, **52**, 2091 (1988).
- 2) D. Koga, A. Isogai, S. Sakuda, S. Matsumoto, A. Suzuki, S. Kimura and A. Ide, *Agric. Biol. Chem.*, **51**, 471 (1987).
- 3) K. J. Kramer and D. Koga, *Insect Biochem.*, **16**, 851 (1986).
- 4) A. Schlumbaum, F. Mauch, U. Vogeli and T. Boller, *Nature*, **324**, 365 (1986).
- 5) R. Vogeli-Lange, A. Hansen-Gehri, T. Boller and F. Meins, Jr., *Plant Science*, **54**, 171 (1988).
- 6) M. A. Raftery, T. Rand-Meir, F. W. Dahlquist, S. M. Parsons, C. L. Borders, R. G. Wolcott, W. Beranek and L. Jao, *Anal. Biochem.*, **30**, 427 (1969).
- 7) T. Tsukamoto, D. Koga, A. Ide, T. Ishibashi, M. Horino-Matsushige, K. Yagishita and T. Imoto, *Agric. Biol. Chem.*, **48**, 931 (1984).
- 8) H. T. S. Britton and R. A. Robinson, *J. Chem. Soc.*, **1931**, 1456.
- 9) D. Koga, M. Nakashima, T. Matsukura, S. Kimura and A. Ide, *Agric. Biol. Chem.*, **50**, 2357 (1986).
- 10) D. Koga, M. S. Mai, C. Dziadik-Turner and K. J. Kramer, *Insect Biochem.*, **12**, 493 (1982).
- 11) D. Koga, J. Jilka and K. J. Kramer, *Insect Biochem.*, **13**, 295 (1983).
- 12) D. Koga, H. Fujimoto, T. Funakoshi, T. Utsumi and A. Ide, *Insect Biochem.*, **19**, 123 (1989).