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Comparative Study of the Reaction Mechanism of Family 18 Chitinases from Plants and Microbes[1]

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Hydrolytic mechanisms of family 18 chitinases from rice (*Oryza sativa* L.) and *Bacillus circulans* WL-12 were comparatively studied by a combination of HPLC analysis of the reaction products and theoretical calculation of reaction time-courses. All of the enzymes tested produced β-anomers from chitin hexasaccharide [$(GlcNAc)_6$], indicating that they catalyze the hydrolysis through a retaining mechanism. The rice chitinases hydrolyzed predominantly the fourth and fifth glycosidic linkages from the nonreducing end of $(GlcNAc)_6$, whereas *B. circulans* chitinase A1 hydrolyzed the second linkage from the nonreducing end. In addition, the *Bacillus* enzyme efficiently catalyzed transglycosylation, producing significant amounts of chitin oligomers larger than the initial substrate, but the rice chitinases did not. The time-courses of $(GlcNAc)_6$ degradation obtained by HPLC were analyzed by theoretical calculation, and the subsite structures of the rice chitinases were identified to be (–4)(–3)(–2)(–1)(+1)(+2). From the HPLC profile of the reaction products previously reported [Terwisscha van Scheltinga *et al.* (1995) *Biochemistry* 34, 15619–15623], family 18 chitinase from rubber tree (*Hevea brasiliensis*) was estimated to have the same type of subsite structure. Theoretical analysis of the reaction time-course for the *Bacillus* enzyme revealed that the enzyme has (–2)(–1)(+1)(+2)(+3)(+4)-type subsite structure, which is identical to that of fungal chitinase from *Coccidioides immitis* [Fukamizo *et al.* (2001) *Biochemistry* 40, 2448–2454]. The *Bacillus* enzyme also resembled the fungal chitinase in its transglycosylation activity. Minor structural differences between plant and microbial enzymes appear to result in such functional variations, even though all of these chitinases are classified into the identical family of glycosyl hydrolases.

Key words: binding subsites, chitinase, chitooligosaccharide, hydrolytic mechanism, theoretical calculation.

Chitinases belong to glycosyl hydrolase families 18 and 19 according to the classification made by Henrissat and Bairoch (*1*). Family 19 chitinases have been found only in plants and some *Streptomyces* strains (*2*), and their structures are similar to that of hen lysozyme and rich in α-helices (*3*). In contrast, family 18 chitinases are distributed in a wide range of organisms, including bacteria, fungi, plants, insects, mammals, and viruses, and have been found to possess a common $(\alpha/\beta)_8$-barrel domain, consisting of eight α-helices and eight β-strands (*4–7*). The catalytic reaction of the family 18 enzymes takes place through a retaining mechanism, in which β-anomers are produced by hydrolysis of β-1,4-glycosidic linkages. It now appears that family 18 chitinases catalyze hydrolysis through the anchimeric assistance of the acetamido group of the substrate sugar residue forming an oxazoline intermediate (substrate-assisted mechanism) (*8*). However, some structural variations are found among the family 18 enzymes. The family 18 chitinase from rubber tree (*Hevea brasiliensis*) consists only of the barrel structure, whereas the family 18 chitinases from microbes, *Serratia marcescence*, *Bacillus circulans* WL-12, and *Coccidioides immitis*, possess minor domains [β-strand rich domain and (α+β) domain] together with the main barrel domain. The β-strand–rich domain of chitinase from *S. marcescence* has been demonstrated to play an important role in the hydrolysis of an insoluble substrate through its binding affinity to the substrates (*9*). Such structural differences between plant and microbial family 18 chitinases might result in differences in their functional properties, such as substrate-binding mode.

Although a number of family 18 chitinase genes have

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Abbreviations GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose, MurNAc, 2-acetamido-3-*O*-(1-carboxyethyl)-2-deoxy-D-glucopyranose, $(GlcNAc)_n$, β-1,4–linked oligosaccharide of GlcNAc with a polymerization degree of *n*, OsChib1a, an acidic family 18 chitinase from *Oryza sativa* L., OsChib1b, a basic family 18 chitinase from *Oryza sativa* L., ChiA1, family 18 chitinase from *Bacillus circulans* WL-12, HPLC, high performance liquid chromatography

been cloned from plant origins, a heterologous expression system of the enzymes has not yet been reported. Recently, the nucleotide sequences of cloned chitinase cDNAs from rice (*Oryza sativa* L.) have been reported, and a *Pichia pastoris* expression system has been found to efficiently produce the rice chitinases (*10–13*) The two class III chitinases from rice [acidic OsChib1a (pI 4.6) and basic OsChib1b (pI 8.2)], which belong to family 18, resemble the rubber tree chitinase in molecular size and amino acid sequences. OsChib1a and OsChib1b share 59 and 68% of identical residues with the rubber tree enzyme, respectively (*11*), suggesting that three-dimensional structures of the rice enzymes would be similar to that of the rubber tree enzyme. Thus, these rice chitinases can be regarded as typical plant family 18 enzymes.

In our initial efforts to establish the relationship between the reaction mechanisms and the molecular structures of the family 18 chitinases, we compared the reaction mechanisms of the rice enzymes and microbial enzymes. Reaction time-courses of the chitin hexasaccharide [$(GlcNAc)_6$] degradation for OsChib1a, OsChib1b, and family 18 chitinase from *Bacillus circulans* WL-12 (ChiA1) were determined by HPLC and then analyzed by use of a theoretical model of the hexasaccharide degradation in which the enzyme is assumed to have a substrate binding cleft consisting of six subsites. Our results revealed clear differences in subsite structure between the rice chitinases and ChiA1. The observed differences were discussed with respect to the structural features, considering the data previously reported for other family 18 enzymes from rubber tree (*H. brasiliensis*) and fungus (*C. immitis*).

MATERIALS AND METHODS

Materials—Chitin oligosaccharides [$(GlcNAc)_n$] were purchased from Seikagaku Kogyo Co. *Pichia* expression kit was the product of Invitrogen Co. A Ni^{2+}-chelating affinity column (His-Trap) was purchased from Pharmacia Biotech. All chemicals used for culture medium were purchased from Difco Laboratories. Other reagents were of analytical grade and commercially available.

Production and Purification of Rice Family 18 Chitinases—Strain construction was as detailed elsewhere (*13*). Briefly, chitinase genes encoding the mature part of acidic and basic class III chitinases (OsChib1a and OsChib1b) were kindly donated by Dr. Yoko Nishizawa, Natl. Inst. Agrobiol Resour., Japan, and fused to the His-tag coding sequence at the 3′-end. The chitinase genes containing the His-tag were expressed in the yeast *Pichia pastoris*, as follows *P. pastoris* strains harbouring the recombinant chitinase genes were grown in YPD (1% yeast extract, 2% pepton, 2% dextrose) for general growth and BMGY (1% yeast extract, 2% pepton, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol) or BMMY (1% glycerol in BMGY is replaced with 1% methanol) medium to induce chitinase gene expression. The fully induced culture was centrifuged to remove cells. The supernatant was adjusted to the final concentrations of 20 mM potassium phosphate (pH 7.6), 10 mM imidazole, and 0.5 M NaCl. After filtration through a membrane filter (0.45 μm, Millipore), the culture supernatant was applied onto a Ni^{2+}-chelating affinity column at a flow rate of 1 ml/min, and then the column was washed with 5 ml of 20 mM phosphate buffer, pH 7.6, containing 10 mM imidazole and 0.5 M NaCl. The adsorbed protein was eluted with the same buffer containing 0.5 M imidazole and 0.5 M NaCl. Fractions containing chitinase activity were pooled and dialyzed against 20 mM phosphate buffer, pH 7.6. The purity was confirmed by SDS–polyacrylamide gel electrophoresis.

Production and Purification of Chitinase A1 from B. circulans WL-12—Chitinase A1 from *B. circulans* WL-12 (ChiA1) was produced and purified by the method previously reported (*14*). The enzyme was produced in *Escherichia coli* HB101 carrying the recombinant plasmid pHT-012. After ammonium sulfate precipitation of the periplasmic protein extract, ChiA1 was purified by chitin affinity column chromatography. The purity of the enzyme was confirmed by SDS–polyacrylamide gel electrophoresis.

HPLC Analysis of Product Anomers—The anomeric form of the enzymatic products was determined by HPLC (*15–17*). The enzymatic hydrolysis of chitin hexasaccharide [$(GlcNAc)_6$] was carried out in 50 mM sodium acetate buffer, pH 5.0 at 25°C. Concentrations of the enzyme and the substrate were 0.3–2.6 μM and 2.7 mM, respectively

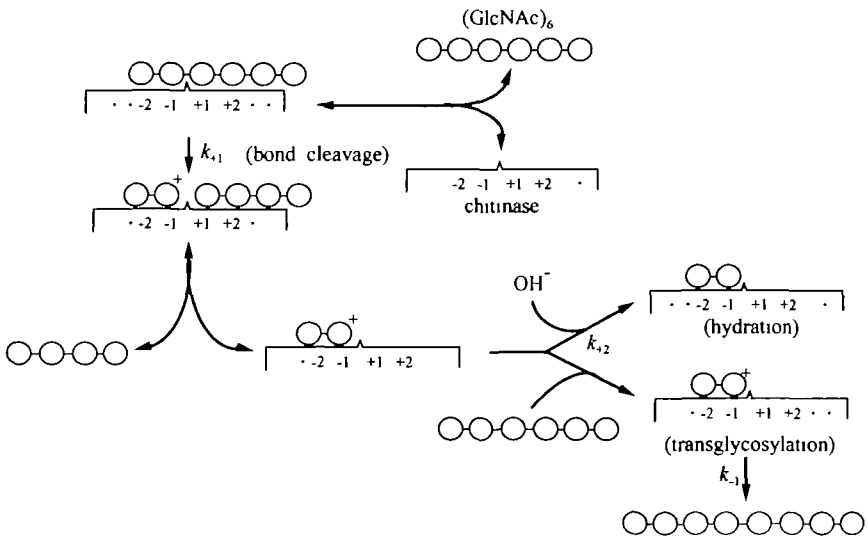

Fig 1 **Theoretical model of the hexasaccharide degradation by chitinase.** In the practical calculation, all of the possible binding modes and possible reaction products are taken into consideration

After incubation for a given period, the enzymatic reaction was terminated by adding an equal volume of 0 5 M NaOH The mixture was then applied onto a TSK Amide 80 column, and elution was performed with 70% acetonitrile at a flow rate of 0.7 ml/min. The initial substrate and the oligosaccharide products were detected by ultraviolet absorption at 220 nm. Splitting mode of the hexasaccharide substrate was qualitatively estimated from the α/β ratio of each oligosaccharide product in the HPLC profiles.

HPLC Determination of the Reaction Time-Course—The reaction products from the chitinase-catalyzed hydrolysis of $(GlcNAc)_6$ were also analyzed by gel filtration HPLC, which gives quantitative data with sufficient accuracy for the following kinetic analysis. The enzymatic reaction was done in 50 mM sodium acetate buffer, pH 5.0, at 40°C. Substrate concentration was 4.9 mM. Enzyme concentration was 0 2–1.1 μM in each experiment The enzymatic reaction was terminated by adding the NaOH solution, and the mixture was applied onto a gel filtration column of TSK-GEL G2000PW (Tosoh). Elution was done with distilled water at a flow rate of 0.3 ml/min. Oligosaccharides were detected as described above.

Theoretical Calculation of the Reaction Time-Course—Theoretical analysis of the reaction time-course was carried out using the reaction model reported for hen egg white lysozyme (Fig. 1) (*18*). A slight modification was introduced into the subsite structure Based on the splitting mode of the substrate estimated from HPLC profiles (Fig. 4), the rice chitinases were assumed to have a (–4)(–3)(–2)(–1)(+1)(+2)-type binding cleft, and ChiA1 a (–2)(–1)(+1)(+2)(+3)(+4)-type binding cleft. To estimate the binding free energy values at individual subsites, an optimization technique based on the modified Powell method (*19*) was employed using the cost function,

$$F = \sum_{i}\sum_{n} [(GlcNAc)^{e}_{n,i} - (GlcNAc)^{c}_{n,i}]^2 \quad (1)$$

where e and c represent the experimental and calculated values, n is the size of the oligosaccharides and i the reaction time. Optimization was conducted by changing the binding free energy values of the individual subsites, while fixing the estimated rate constant values. The rate constant value of k_{+1} (for cleavage of glycosidic linkage) was assumed to be dependent upon the substrate size (*20–22*) and was estimated from the initial velocity data. The value of k_{-1} (rate constant for transglycosylation) for the rice chitinases was estimated to be zero, because no transglycosylation product was detected in the HPLC profile. The k_{-1} value for ChiA1 was estimated on the basis of the value reported for *C. immitis* chitinase (*22*). At present, it is difficult to determine accurately the value of k_{+2} (rate constant for hydration). As described by Masaki *et al.* (*18*), however, the value of k_{-1}/k_{+2}, which represents the relative efficiency of transglycosylation, could be estimated from the reaction time-course. Thus, a constant value of 200.0 s^{-1} was tentatively allocated to k_{+2} in each chitinase reaction, as in the case of *C. immitis* chitinase (*22*), and the k_{-1}/k_{+2} value was calculated from the estimated k_{-1} value. Discussion was based only on the k_{-1}/k_{+2} value, not on the absolute values of the individual rate constants.

RESULTS

Anomer Formation and Splitting Mode of Chitinases—To investigate the splitting mode of OsChib1a, OsChib1b, and ChiA1, we first analyzed the anomer formation from $(GlcNAc)_6$ substrate by HPLC. As shown in Fig. 2, at the initial reaction stage, OsChib1a produced predominantly β-$(GlcNAc)_4$, which mutarotated spontaneously to α-$(GlcNAc)_4$. The α/β ratio of $(GlcNAc)_2$ produced by OsChib1a was rather close to that at mutarotation equilibrium (α/β = 1.6). OsChib1b produced β-$(GlcNAc)_5$ together with β-$(GlcNAc)_4$, while the α/β ratios of GlcNAc and $(GlcNAc)_2$ produced were close to the equilibrium value over the entire course of the reaction. These results indicate that both enzymes catalyze an anomer retention reaction, but at different glycosidic linkages. OsChib1a appears to split preferentially the fourth linkage from the nonreducing end of $(GlcNAc)_6$, whereas OsChib1b splits the fourth and fifth linkages with equal efficiency. The splitting modes estimated from these HPLC profiles are shown in Fig. 4

In contrast, ChiA1 produced a large amount of β-$(GlcNAc)_2$ in the early stage of the reaction (Fig 3). The α/β ratio of $(GlcNAc)_4$ produced was nearly at equilibrium. Thus, ChiA1 appears to hydrolyze predominantly the second glycosidic linkage from the nonreducing end of the hexasaccharide substrate. The splitting mode is also shown in Fig. 4.

Time-Course of the Chitinase Reaction Determined by Gel Filtration HPLC—As shown in Fig. 5A, OsChib1a predominantly produced $(GlcNAc)_2$ and $(GlcNAc)_4$ from $(GlcNAc)_6$, and the generated $(GlcNAc)_4$ was slowly decomposed further to $(GlcNAc)_2$ OsChib1b produced all possible oligosaccharide products from $(GlcNAc)_6$, from GlcNAc to $(GlcNAc)_5$ (Fig. 6A), and the product distribution was much more complicated than that obtained with OsChib1a. However, the splitting to $(GlcNAc)_5$+GlcNAc appeared to be predominant, and $(GlcNAc)_5$ produced was immediately hydrolyzed further into $(GlcNAc)_4$+GlcNAc or $(GlcNAc)_3$+$(GlcNAc)_2$. No transglycosylation product was detected with these rice chitinases. ChiA1 (Fig 7A) predominantly produced $(GlcNAc)_2$ and $(GlcNAc)_4$ from $(GlcNAc)_6$, and a significant amount of transglycosylation product was also detected, which was identified from its retention time as $(GlcNAc)_8$. After a fraction of $(GlcNAc)_6$ has been hydrolyzed into $(GlcNAc)_2$+$(GlcNAc)_4$, the intact $(GlcNAc)_6$ is thought to join with the enzyme-bound $(GlcNAc)_2$, of which the reducing end C1 carbon is in a transition state, producing $(GlcNAc)_8$ (Fig. 1).

Estimation of Binding Free Energy Changes of the Subsites—Based on the splitting modes of the hexasaccharide substrate (Fig. 4), the subsite structure of the rice chitinases was predicted to be (–4)(–3)(–2)(–1)(+1)(+2), and this was employed for the theoretical calculation of the reaction time-courses for the rice enzymes Since OsChib1a hydrolyzed $(GlcNAc)_6$ predominantly into $(GlcNAc)_4$+$(GlcNAc)_2$, the hexasaccharide bound on the enzyme exclusively occupies all of the six subsites. The population of the enzyme-$(GlcNAc)_6$ complexes having a vacant subsite should be very small in this case. Thus, the sugar-residue affinities at both ends of the subsites should be higher than those at the internal subsites. Furthermore, since the catalysis takes place through a substrate-assisted mechanism, the free energy at site (–1) should have an unfavorable positive value, as in the case of *C. immitis* chitinase (+3.1 kcal/mol) (*22*). Such an unfavorable value reflects the complementarity of the oxazoline-like intermediate at this subsite. Based

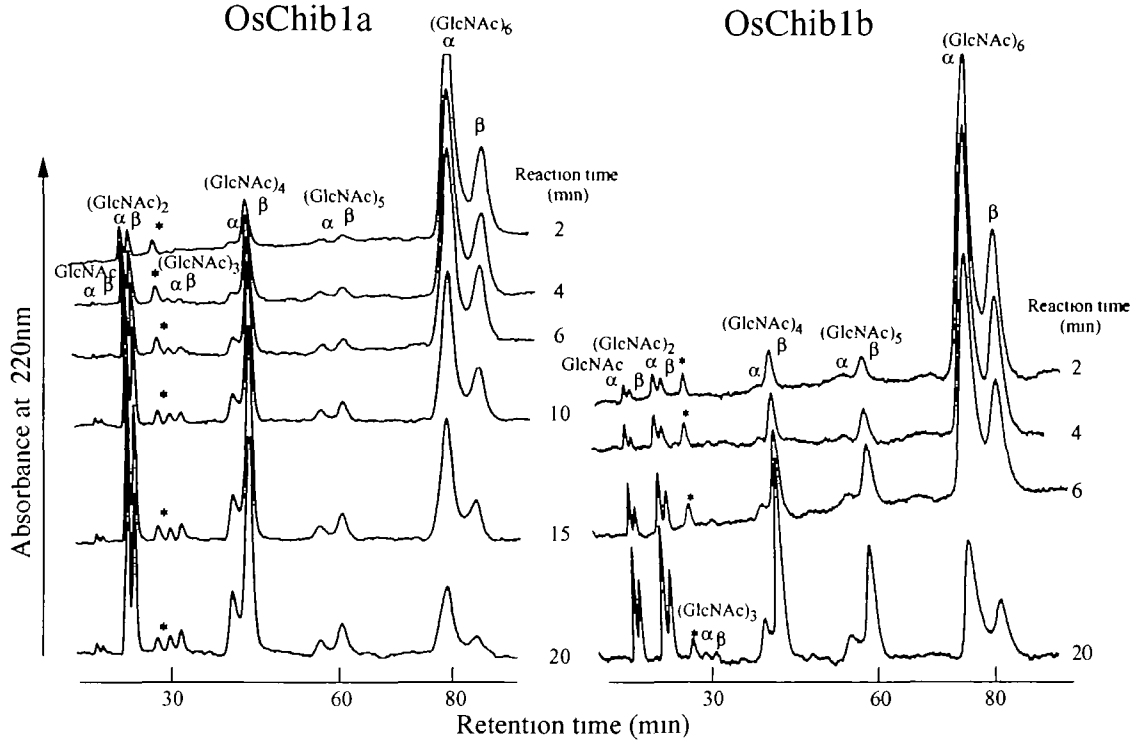

Fig 2 **Anomer formation catalyzed by rice family 18 chitinases from the hexasaccharide substrate.** Left panel, OsChib1a (0 3 μM), Right panel, OsChib1b (2 1 μM) Each enzyme was added to 4 9 mM $(GlcNAc)_6$ solution in 50 mM sodium acetate buffer, pH 5 0, and incubated at 25°C for a given period HPLC conditions are described in the text The peaks designated by * are derived from impurity

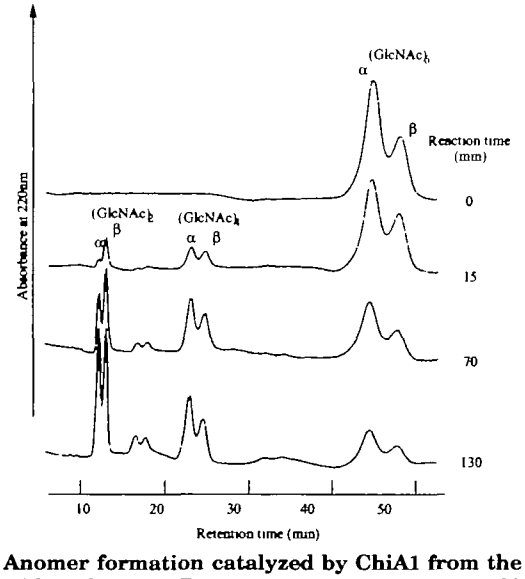

Fig 3 **Anomer formation catalyzed by ChiA1 from the hexasaccharide substrate.** Enzyme concentration was 0.3 μM Reaction and HPLC conditions are the same as in Fig. 2

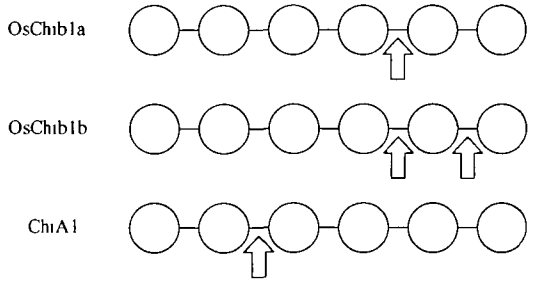

Fig 4 **Splitting mode of the hexasaccharide substrate by the three chitinases.** Arrows indicate the splitting position by each chitinase

on these considerations, we first tentatively allocated the values, –2.0, –1.0, 0.0, +3.1, –1.0, and –2.0 kcal/mol, to the binding free energy changes at the individual subsites of (–4)(–3)(–2)(–1)(+1)(+2), respectively, and conducted the time-course calculation. However, the calculated time-course did not fit the experimental one. In particular, the rate of $(GlcNAc)_6$ degradation in the calculated time-course was much higher than the observed rate. Thus, the time-course calculation was repeated with a reduced affinity, –1.5 kcal/mol, for both ends of the subsites, (–4) and (+2), resulting in improved fitness between the calculated and experimental time-courses. Starting from these initial estimates, –1.5, –1.0, 0.0, +3.1, –1.0, and –1.5 kcal/mol for the subsites from (–4) to (+2), optimization was carried out by changing the free energy values, while fixing the rate constant values. Finally, we obtained the optimized free energy values of –1 5 ± 0.2, –1.3 ± 0.4, +0.2 ± 0.2, +3 1 ± 0.2, –0.4 ± 0.1, and –1.7 ± 0.1 kcal/mol for the corresponding subsites of OsChib1a. The calculated time-course giving the best fit is shown in Fig. 5B.

The fact that OsChib1b produced $(GlcNAc)_{1-5}$ from $(GlcNAc)_6$ indicates that splitting is possible at multiple glycosidic bonds, and hence several binding modes of the hexasaccharide substrate should be considered for this enzyme. In this case, the affinities at both ends of the subsites

should be lower than those at the internal subsites. Site (–1) should have an unfavorable free energy value, +3 1 kcal/mol, as in the case of OsChib1a. Thus, we at first conducted the time-course calculation with the free energy values of 0.0, –1.0, –5.0, +3.1, –1.0, and 0.0 kcal/mol for the individual subsites (–4)(–3)(–2)(–1)(+1)(+2), respectively In the calculated time-course, however, the frequency of splitting to $(GlcNAc)_5$+GlcNAc was identical to that to $(GlcNAc)_4$+$(GlcNAc)_2$, and the splitting to $(GlcNAc)_3$+$(GlcNAc)_3$ was much higher than that found experimentally. We then calculated the time-course with a reduced value, +0.5 kcal/mol, at site (+2) to enhance the splitting to $(GlcNAc)_5$ +GlcNAc, and with an increased value, –1.0 kcal/mol, at site (–4) to suppress the splitting to $(GlcNAc)_3$+$(GlcNAc)_3$ This calculation gave a much better fit to the experimental values. Thus, we used values of –1.0, –1.0, –5.0, +3.1, –1.0, and +0.5 kcal/mol as the initial estimates for optimization. Finally, the values of –1.0 ± 0.2, –0.5 ± 0.1, –5.0 ± 0 5, +3.1 ± 0.2, –1.5 ± 0.1, and +0.5 ± 0.1 kcal/mol were found to be optimal for the corresponding subsites of OsChib1b As shown in Fig 6B, the calculated time-course is satisfactorily similar to the experimental profile, except that the amount of $(GlcNAc)_2$ produced was slightly smaller than that found experimentally

In the case of ChiA1, the profile of the experimental time-course (Fig. 7A) was similar to that of *C. immitis* chitinase (*22*) Thus, we adopted the subsite model used for *C immitis* chitinase, (–2)(–1)(+1)(+2)(+3)(+4). The rate constant of transglycosylation (k_{-1}) was first assumed to be the same as that estimated for *C. immitis* chitinase (220 s^{-1}), and the optimization was conducted using the free energy values estimated for *C. immitis* chitinase, –3.8, +3.1, –2.5, –3 0, +0.8, and –1.8 kcal/mol, as the initial estimates. However, the value of the cost function obtained by the optimization was higher than those obtained so far. The fitness was not satisfactory, especially in the amount of transglycosylation product, $(GlcNAc)_8$. Thus, we tried to change the k_{-1} value to improve the fitness in the amount of $(GlcNAc)_8$ produced. Then we found that 180 s^{-1} is the most appropriate for the k_{-1} value of ChiA1. The optimization was again conducted using this k_{-1} value, and finally we obtained the optimized free energy values of –3.1 ± 0.1, +3.5 ± 0.1, –2.5 ± 0.2, –2.3 ± 0.4, +0.8 ± 0.3, and –1.6 ± 0.2 kcal/mol for ChiA1, and the best-fitted time-course is shown in Fig. 7B. All of these values of kinetic parameters for the chitinases are listed in Table I together with the values for *C immitis* chitinase.

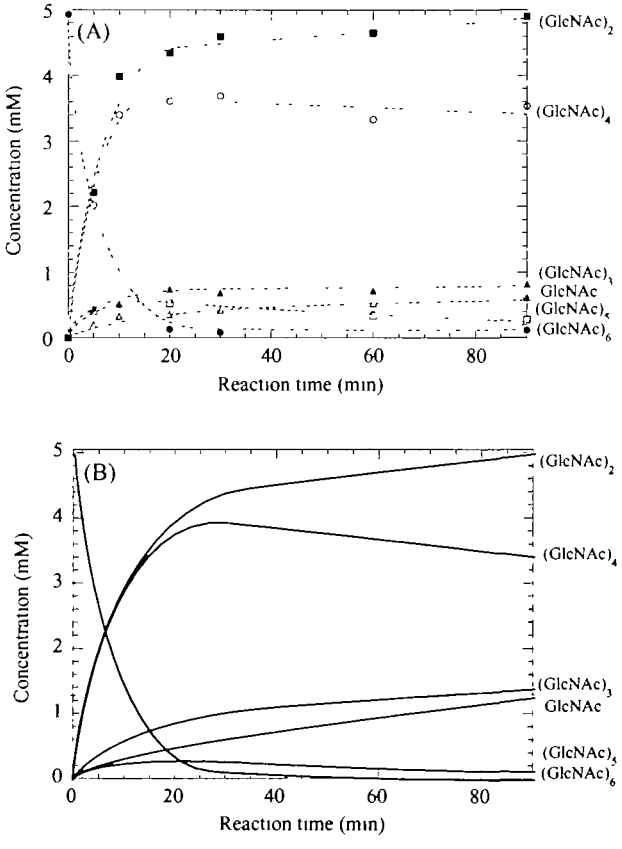

Fig 5. **Time-course of hexasaccharide degradation by OsChib1a.** (A) Experimental time-course. Enzyme concentration was 0 2 μM Reaction conditions were the same as in Fig 2, except that the reaction temperature was set at 40°C. The broken lines were drawn by roughly following the experimental data points. Symbols are as follows, △, GlcNAc; ■, $(GlcNAc)_2$, ▲, $(GlcNAc)_3$, ○, $(GlcNAc)_4$, □, $(GlcNAc)_5$, ●, $(GlcNAc)_6$, ◇, $(GlcNAc)_8$ (B) Theoretical time-course The values of the reaction parameters used for the calculation are listed in the first row of Table I

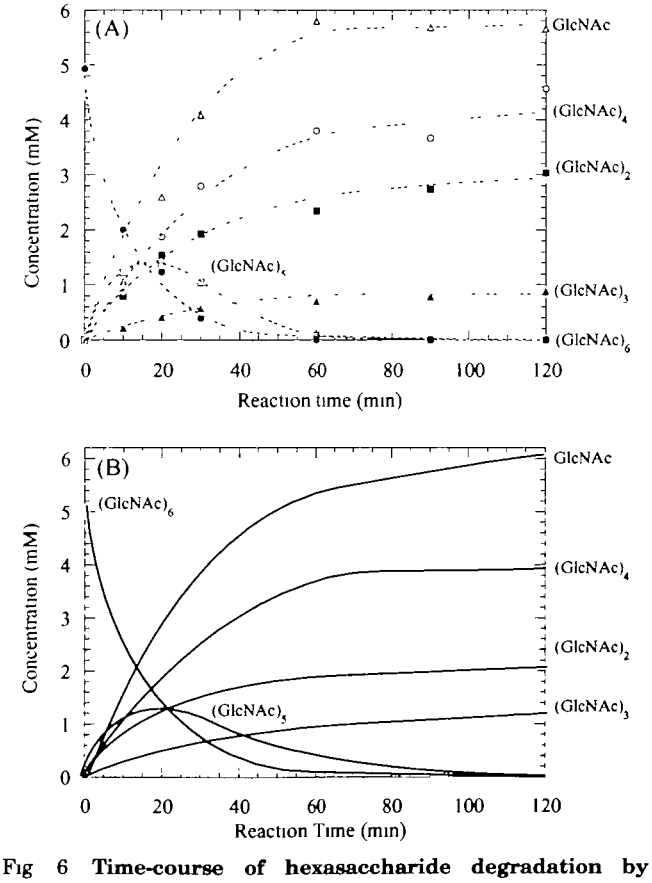

Fig 6 **Time-course of hexasaccharide degradation by OsChib1b.** (A) Experimental time-course. Enzyme concentration was 1 1 μM. Reaction conditions were the same as in Fig 5 The broken lines were drawn by roughly following the experimental data points Symbols are the same as in Fig 5 (B) Theoretical time-course The values of the reaction parameters used for the calculation are listed in the second row of Table I

DISCUSSION

It is generally accepted that family 18 chitinases catalyze the hydrolytic reaction through a substrate-assisted mechanism, in which the anomeric form of the glycosidic linkage is retained after hydrolysis (*8, 23, 24*). In this study, we confirmed the accepted mechanism with the family 18 chitinases from rice and *B. circulans* WL-12: the anomeric form is retained in the catalytic reaction of the rice chitinases (OsChib1a and OsChib1b) and ChiA1 (Figs. 2 and 3). On the other hand, anomer analysis of the product by HPLC afforded additional information: the β-form is abundant at a newly produced reducing end, whereas the α/β ratio is close to equilibrium at an original reducing end. Thus, the HPLC separation of anomeric forms of the reaction products allows us to estimate the splitting mode of an oligosaccharide substrate (*15, 17*). The splitting modes of OsChib1a and OsChib1b were found to differ greatly from that of ChiA1 (Fig. 4). The rice chitinases preferentially hydrolyze the fourth or fifth glycosidic linkage from the nonreducing end of the hexasaccharide substrate, whereas ChiA1 hydrolyzes the second linkage. This splitting mode of ChiA1 confirms the results previously obtained with *p*-nitrophenyl *N*-acetyl-β-chitooligosaccharides as substrates (*25*). It should be noted that the splitting mode of ChiA1 is similar to that of fungal chitinase from *C. immitis* (*22*).

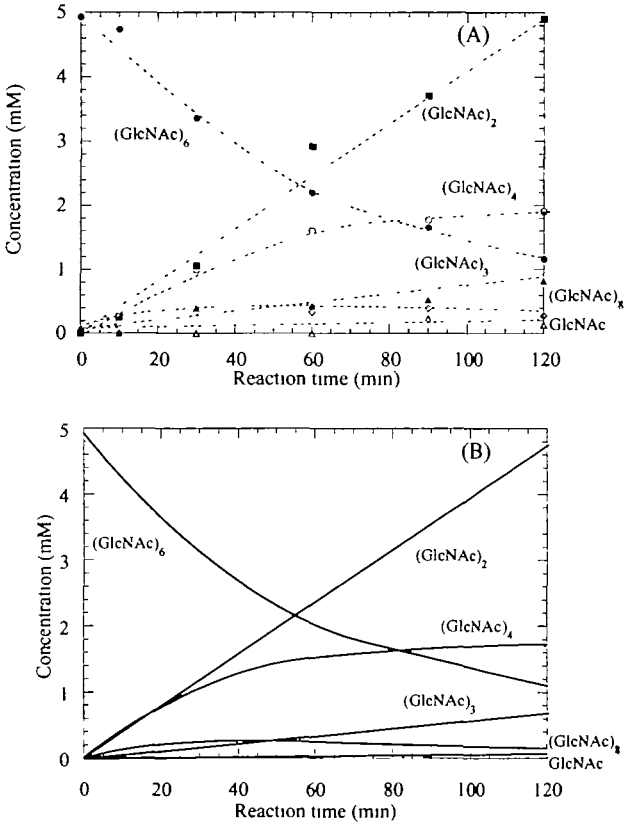

Fig 7 **Time-course of hexasaccharide degradation by ChiA1.** (A) Experimental time-course. Enzyme concentration was 0 3 μM Reaction conditions were the same as in Fig. 5 The broken lines were drawn by roughly following the experimental data points Symbols are the same as in Fig 5 (B) Theoretical time-course. The values of the reaction parameters used for the calculation are listed in the third row of Table I

Such a difference in splitting mode must result from the subsite structure in the binding cleft To study further the difference between the plant and microbial family 18 enzymes, we conducted a theoretical analysis of the time-courses of hexasaccharide degradation using the model shown in Fig. 1, and obtained the free energy distribution in the substrate binding cleft. In this model, we assumed that the rate constant of bond cleavage is dependent upon the substrate size. Such an assumption seems to be essential, because the bond cleavage rate is cooperatively affected by the substrate occupancy of the subsites, which depends on the substrate size. The validity of the assumption is confirmed by the fact that the profiles of theoretical time-courses were in good agreement with those obtained experimentally (Figs. 5, 6, and 7). As Table I shows, the binding clefts of the plant enzymes extend to the nonreducing end (negatively numbered subsites), whereas those of the microbial enzymes extend to the opposite side (positively numbered subsites). Family 18 chitinase from rubber tree (*H. brasiliensis*) produces the β-anomer of $(GlcNAc)_4$ from the $(GlcNAc)_5$ substrate (*23*), indicating that $(GlcNAc)_5$ predominantly binds to subsites (–4)(–3)(–2)(–1)(+1) of the rubber tree enzyme, which produces $(GlcNAc)_4$+GlcNAc. From the free energy distribution in OsChib1b, $(GlcNAc)_5$ is estimated to preferentially bind to subsites from (–4) to (+1), suggesting that the rubber tree chitinase might have a free energy distribution similar to that of OsChib1b.

TABLE I **Kinetic parameter values of four family 18 chitinases.**

		Rate constants (s^{-1})				Binding free energy change (kcal/mol)							
		k_{+1}	k_{-1}	k_{+2}	k_{-1}/k_{+2}	(–4)	(–3)	(–2)	(–1)	(+1)	(+2)	(+3)	(+4)
	$(GlcNAc)_6$	48,000											
OsChib1a	$(GlcNAc)_5$	32,000	0 0	200 0	0	–1 5	–1 3	+0 2	+3 1	–0 4	–1 7		
	$(GlcNAc)_4$	32,000											
	$(GlcNAc)_6$	60											
OsChib1b	$(GlcNAc)_5$	66	0 0	200 0	0	–1.0	–0 5	–5 0	+3 1	–1 5	+0 5		
	$(GlcNAc)_4$	6											
	$(GlcNAc)_6$	54											
ChiA1	$(GlcNAc)_5$	45	180.0	200 0	0 9			–3 1	+3.5	–2 5	–2 3	+0 8	–1.6
	$(GlcNAc)_4$	16											
Coccidioides immitis[a]	$(GlcNAc)_6$	0 5											
	$(GlcNAc)_5$	0 6	220.0	200 0	1.1			–3 8	+3 1	–2 5	–3 0	+0 8	–1.8
	$(GlcNAc)_4$	1 7											

[a]From Ref. *21*.

It is noteworthy that a significant activity of transglycosylation is found in the microbial enzymes but not in plant enzymes. Hen egg white lysozyme has been recognized to efficiently catalyze the glycosyl transfer (*26, 27*). Kinetic analysis of the lysozyme reaction toward oligosaccharide substrate indicated that the ratio of the rate constant of transglycosylation (k_{-1}) to that of hydration (k_{+2}) is 133 (*18*). In this study, the k_{-1}/k_{+2} ratios of the microbial chitinases were found to be about 1 (Table I). Nevertheless, the microbial enzymes catalyze the glycosyl transfer as efficiently as the lysozyme does The transglycosylation efficiency might be controlled not only by the rate constant value of k_{-1} but also by the acceptor-binding ability. The acceptor-binding site of hen egg white lysozyme is composed of subsites (+1) and (+2) The acceptor-binding site (positively numbered subsites) of ChiA1 extends farther than that of hen egg white lysozyme. This situation might explain the high transglycosylation efficiency of the microbial chitinases.

OsChib1a and OsChib1b respectively share 59 and 68% of the identical amino acid residues with the chitinase from rubber tree (*H. brasiliensis*), and the substrate-binding residues proposed for the rubber tree chitinase (*5, 23*) are mostly conserved in the rice chitinases, the exceptions being those corresponding to Asn34 and Ile82 of the rubber tree enzyme, which are located at subsites (–3) and (–2), respectively (*23*). Asn34 is replaced by Ser in OsChib1a and by Pro in OsChib1b, while Ile82 is replaced by Ala in OsChib1a and by Val in OsChib1b Since the binding free energy changes at these sites differ significantly between OsChib1a and OsChib1b (Table I), these substitutions might be responsible at least partly for the different cleavage profiles of the rice enzymes (Figs. 5 and 6). The different cleavage profiles might also derive from the free energy differences at sites (+1) and (+2). However, information on these subsites is very limited, even in the rubber tree chitinase. Thus, further discussion on this point is impossible at present. Itoh and his coworkers reported that OsChib1b exhibits lytic activity against *Micrococcus lysodeikticus*, but OsChib1a does not (*11*). OsChib1b would have a stronger affinity and a higher k_{+1} value toward a (GlcNAc-MurNAc)$_n$-type substrate than a (GlcNAc)$_n$-type substrate.

Watanabe and his coworkers (*7, 28*) reported the crystal structure of the catalytic domain of ChiA1 complexed with (GlcNAc)$_7$, in which (GlcNAc)$_7$ binds to the substrate binding cleft composed of (–5)(–4)(–3)(–2)(–1)(+1)(+2). They suggested that essential aromatic residues for binding and hydrolyzing crystalline chitin are present at the sites (–5) to (–3). Their suggestion appears to be inconsistent with the data obtained in this study. Since the cocrystal was obtained with an inactive mutant, E204Q, the complex is stable and nonproductive. In the productive complex of the active enzyme with the substrate, however, a complicated motion of the bound substrate, such as a processivity, might be possible, which is suppressed in the nonproductive complex. This situation might have resulted in the inconsistency between the subsite structures proposed.

Overall sequence similarity between ChiA1 and the rice chitinases is very low. When gaps were introduced into the rice chitinases to align the sequences, homologous sequences were found in the catalytic residue region, but not in the substrate-binding region. Absence of the subsites (–4) and (–3) in ChiA1 (Table I) might be related to such substitutions in the substrate-binding region. A minor domain, such as a β-rich domain, present in ChiA1 might participate in the formation of subsites (+3) and (+4), resulting in the high efficiency of transglycosylation activity. Fusion protein genes are now under construction using the chitinase genes from plant and fungus, and the fusion proteins expressed should be characterized to identify the structural factors controlling the enzyme function.

In conclusion, the plant family 18 chitinases have a (–4)(–3)(–2)(–1)(+1)(+2)-type binding cleft, while the microbial family 18 chitinases a (–2)(–1)(+1)(+2)(+3)(+4)-type binding cleft. Transglycosylation activity is found in the latter, but not in the former. Further studies are needed to confirm if the functional differences are universally observed between the plant and microbial family 18 enzymes.

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