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# Characterization of Monoclonal Antibodies to Chitinase A1 and Enhancement of Chitinase A1 Activity by Monoclonal Antibodies

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**SUMMARY**: A total of eleven hybridomas which secrete antibodies against chitinase A1 were established. Among the eleven monoclonal antibodies (MAbs) obtained, six recognized the catalytic domain, three recognized the type III region and two recognized the chitin binding domain of chitinase A1. Of the two MAbs which recognized the chitin binding domain one was found to also react with chitinase D, but none of the other MAbs which recognized either the type III region or the chitin binding domain reacted with chitinase D despite the extensive amino acid sequence similarity between both the type III and chitin binding domains of the two chitinases. Two of the eleven MAbs enhanced chitinase activity significantly, while the other MAbs did not have any significant effect on chitinase A1 activity. 

Next to cellulose, chitin is the second most abundant polysaccharide in nature. Bacterial chitinases are thought to play a major role in its degradation, converting it into a biologically useful form for recycling in nature. We have been studying the chitinase system of *Bacillus circulans* WL-12 in order to better understand the mechanisms underlying chitin degradation by bacteria. More than six distinct chitinase molecules have been detected in culture supernatants of this bacterium when grown in the presence of chitin (1). Among these chitinases, chitinase A1 is believed to be the key enzyme in the chitinase system as it is the chitinase most abundantly produced, and the one with the highest colloidal chitin hydrolyzing activity. In addition it has a strong affinity to insoluble substrate, chitin. Structurally, the mature form of chitinase A1 consists of four discrete functional domains, namely, the catalytic domain, two type III modules (domains) and a C-terminal chitin binding domain. The type III modules, R-1 and R-2, have been found to be homologous to the so-called type III repeats of fibronectin, a multifunctional extracellular matrix and plasma protein of higher eukaryotes (2). The C-terminal chitin binding domain (CA) and type III domains are important for efficient hydrolysis of insoluble chitin (3).

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In order to study binding properties and binding mechanism of the enzyme to chitin, it is useful to isolate and analyze the chitin binding domain and type III domains separately from the catalytic domain. Monoclonal antibodies (MAbs) specific to each domain would facilitate in the identification and purification of such domains. To this end, we attempted to isolate MAbs specific to each domain of chitinase A1 and this paper describes the isolation and characterization of 11 monoclonal antibodies directed against chitinase A1.

## MATERIALS AND METHODS

***Production and purification of chitinases***

Intact chitinase A1 and five modified chitinase A1 derivatives having various deletions at their C-terminal chitin binding domains and/or type III modules were produced in *E. coli*. The purification of intact chitinase A1 was described in a previous report (4). Crude enzyme preparations of the five modified chitinase A1 proteins were prepared by 60% ammonium sulfate precipitation and used without further purifications.

Intact chitinase D was produced in *E. coli* HB101 harboring the recombinant plasmid pHTDR15 which carries the *chiD* gene from *Bacillus circulans* WL-12 (5). A modified chitinase D lacking the type III module was produced in *E. coli* harboring plasmid pHTDR25(ΔIII) which carries the mutant *chiD* gene lacking the region corresponding to the type III module. Plasmid pHTDR25(ΔIII) was constructed from plasmid pHTDR15 by removing a 279 bp *Acc* III-*Nsp* V fragment corresponding to the type III domain. *E. coli* HB101 carrying the plasmid encoding either intact (pHTDR15) or modified (pHTDR25) *chiD* gene was grown in the L-broth medium containing 100 μg/ml ampicillin for 24 h at 30 °C. Cells were collected by centrifugation and periplasmic proteins were extracted from the cells by the osmotic shock procedure of Manoil and Beckwith (6). Ammonium sulfate was added to the extract to achieve 60% saturation and the resulting precipitate was dissolved in a small volume of 10 mM succinate buffer (pH 6.0), dialyzed overnight against the same buffer and lyophilized. The crude chitinases were then dissolved in a small volume of 1 mM sodium phosphate buffer, pH 6.0, and applied on hydroxyapatite column (3.9 x 6 cm) equilibrated with the same buffer. Elution was carried out with the same buffer and the unadsorbed protein fraction containing purified chitinases were collected and lyophilized.

***Production of monoclonal antibodies (MAbs)***

Purified chitinase A1 (100 μg) mixed with an equal volume of complete Freund's adjuvant was injected subcutaneously twice at a 1 week interval into female BALB/c mice. After the second injection, one intraperitoneal booster injection of 100 μg of chitinase A1 without adjuvant was given and the spleen was removed 3 days later. Splenic lymphocytes were collected and fused with the myeloma cell line P3-NS1-Ag4-1 (7). Myeloma and hybridoma cells were cultured in RPMI 1640 medium (Nissui pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 2 mM glutamine and 10% fetal calf serum (GIBCO BRL, Giathersburg, USA). Fusions were performed in RPMI 1640 medium and hybridomas were grown on mouse-peritoneal macrophage feeders with selection by the addition of medium containing hydroxylamine and thymidine (8). Culture supernatants were tested for antibody by ELISA as described below. Positive hybridomas secreting specific antibody were cloned at least twice by limiting dilution. The established hybridomas lines were cultured in RPMI medium containing 10% fetal calf serum or injected intraperitoneally into pristane-treated BALB/c mice to produce ascites. MAbs were isolated from the ascites fluid by 50% ammonium sulphate precipitation followed by affinity chromatography on a protein A-Sepharose column. Purified IgG concentration was determined spectrophotometrically using $E^{1\%}_{280\ nm}$ =14.0. The immunoglobulin isotype of each hybridoma line was determined with isotype-specific antisera (ICN ImmunoBiologicals, Lisie, USA) by double immunodiffusion in agar.

***ELISA***

After addition of 50 μl of the hybridoma culture supernatant to wells coated with purified chitinase A1(3 μg/ml), the microtiter plates were incubated at 37°C for 30 min. and then washed 4 times with 0.01M phosphate buffer (pH 7.2) containing 0.05%

Tween 20. To each well 50 μl of a 1:5000 dilution of goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (DAKO A/S, Copenhagen, Denmark) in 0.01M phosphate buffer, pH 7.2, was added. The plates were incubated for 30 min. at 37 °C and washed 4 times with 0.01M phosphate buffer, pH 7.2, containing 0.05% Tween 20. Finally 100 μl of substrate solution containing 0.02% $H_2O_2$ and *o*-phenylenediamine (3 mg/ml) was added and the plates were incubated at room temperature in the dark for 30 min. with the reaction terminated by the addition of 100 μl of 2N $H_2SO_4$. The absorbance was read at 490 nm using a microplate reader (SJeia autoreader, Sanko Junyaku Co., Ltd., Osaka, Japan).

***Electrophoresis and Western blotting***

Proteins were separated in 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) (9) and transferred electrophoretically onto a nitrocellulose membrane using a trans-blot apparatus (AE-6675. P/N, Atto Co., Ltd. Tokyo, Japan). The nitrocellulose membrane was then saturated with phosphate buffered saline (PBS) containing 0.5% skimmed milk and 0.05% Tween 20 (PBS-ST) for 1 h at room temperature, cut in strips, and soaked with a 1:100 dilution of the hybridoma culture supernatant for 1 hr in PBS-ST. After washing with PBS-ST, the nitrocellulose strips were incubated for 1 h at room temperature with a 1:1000 dilution of biotinylated horse anti-mouse IgG (H+L) (Vector Laboratories, Inc., Burlingame, USA) in PBS-ST. The nitrocellulose strips were washed three times with PBS-ST, and incubated for 10 min. at room temperature with a 1:2000 dilution of streptavidin conjugated with alkaline-phosphatase (GIBCO BRL, Gaithersburg, USA) in PBS containing 10% bovine serum albumin. After washing with PBS containing 0.05% Tween 20 and then with 0.1M Tris buffer (pH 9.5) containing 20 mM $MgCl_2$, staining was performed with Bromo-chloro indoylphosphate and Nitroblue tetrazolium.

***Protein determination***

Protein concentration was estimated by the method of Bradford (10) using bovine serum albumin as standard.

***Chitinase activity***

Purified chitinase A1 (10 μl, 5 μg) was mixed with either 50 μl of hybridoma culture supernatant or purified MAbs from ascites fluid, and incubated for 30 min. at 25 °C. 1.0 ml of 0.1 mM *p*-nitrophenyl-*N*,*N*',*N*"-triacetyl chitotriose [*p*NP-$(GlcNAc)_3$](Yaizu Suisan Industry Co., Ltd. Yaizu, Japan) in 0.1M phosphate buffer (pH6.0) was then added and the mixture was incubated at 37 °C. The absorbance at 337 nm ($A_{337nm}$) was monitored and chitinase activity was expressed as the initial rate of increase in $A_{337nm}$ ($\Delta A_{337nm}$/min).

## RESULTS AND DISCUSSION

***Production of MAbs against chitinase A1 and antigenic epitopes for these antibodies***

A total of 11 hybridomas which secreted antibodies against chitinase A1 were established. The isotypes of these antibodies were identified as IgG (Table 1) and the antigenic epitopes they recognize were analyzed by Western blotting against intact and the five modified chitinase A1 molecules produced in *E. coli*. The domain organization of intact and modified chitinase A1 constructs are schematically shown in Fig.1 and include chitinases lacking the following regions: one type III 1module (ChiA1Δ III), two type III modules (ChiA1Δ2III), the C-terminal chitin binding domain (ChiA1ΔCA), the C-terminal domain plus one type III module (ChiA1Δ IIICA) and the C-terminal domain plus two type III modules (ChiA1Δ2IIICA).

Monoclonal antibodies (MAbs) H-7-6M, G-23-3M, G-2-5M, 2C-2-12M, 2F-31-9M and 2E-10-3M were found to react with chitinase A1 (ChiA1) and all modified chitinases (Table 1). Since the region common to all these chitinases is the catalytic domain, we

Table 1. Characterization of monoclonal antibodies against chitinase A1

| Monoclonal antibody | Subclass of Ig | Western-blot reactivity | | | | | |
|---|---|---|---|---|---|---|---|
| | | ChiA1 | ChiA1⊿CA | ChiA1⊿Ⅲ | ChiA1⊿ⅢCA | ChiA1⊿2Ⅲ | ChiA1⊿2ⅢCA |
| H-7-6M | IgG1 | + | + | + | + | + | + |
| G-23-3M | IgG1 | + | + | + | + | + | + |
| G-2-5M | IgG1 | + | + | + | + | + | + |
| 2C-2-12M | IgG1 | + | + | + | + | + | + |
| 2F-31-9M | IgG1 | + | + | + | + | + | + |
| 2E-10-3M | IgG1 | + | + | + | + | + | + |
| G-3-20M | IgG2a | + | + | - | + | - | - |
| D-31-5M | IgG2b | + | + | + | + | - | - |
| 2D-20-5M | IgG2b | + | + | - | - | - | - |
| 2B-11-5M | IgG2a | + | - | + | - | + | - |
| 2F-13-2M | IgG2b | + | - | + | - | + | - |

concluded the MAbs recognize the catalytic domain of chitinase A1. The results obtained with G-2-5M, an example of this type, is shown in Fig. 2B.

As shown in Fig. 2C, MAb G-3-20M reacted with ChiA1, ChiA1ΔCA and ChiA1ΔIIICA, but not with ChiA1ΔIII, ChiA1Δ2III, or ChiA1Δ2IIICA. As the modified *chiA* gene encoding ChiA1ΔIII was constructed by removing the PstI-PstI fragment in the type III region (see Fig.1), the type III module in ChiA1ΔIII is a hybrid

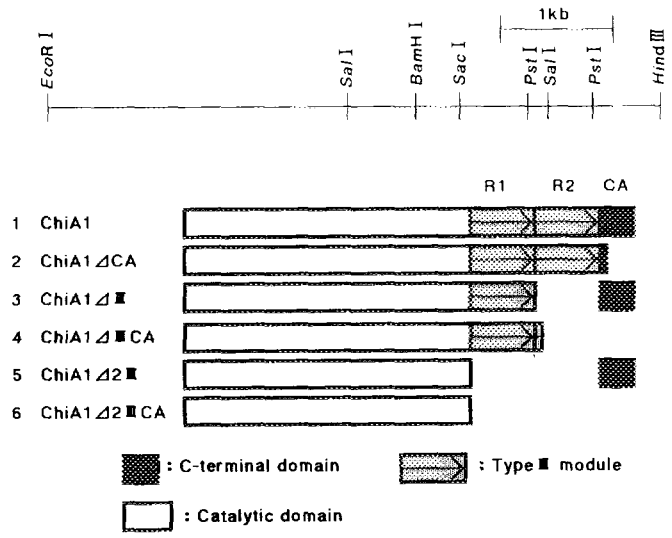


**Fig. 1. Domain organization of deletion derivatives of chitinase A1 encoded by the modified *chiA* genes.** Numbers 1 through 6 correspond to intact chitinase A1 (ChiA1), chitinase A1 lacking C-terminal chitin binding domain (ChiA1ΔCA), one type III module(R-1) (ChiA1ΔIII), one type III module(R-2) plus C-terminal domain (ChiA1ΔIIICA), both type III module (ChiA1Δ2III) and two type III modules plus C-terminal domain (ChiA1Δ2IIICA), respectively. Restriction map of the inserted DNA in the plasmid pHT012 carrying intact *chiA* gene is also shown.

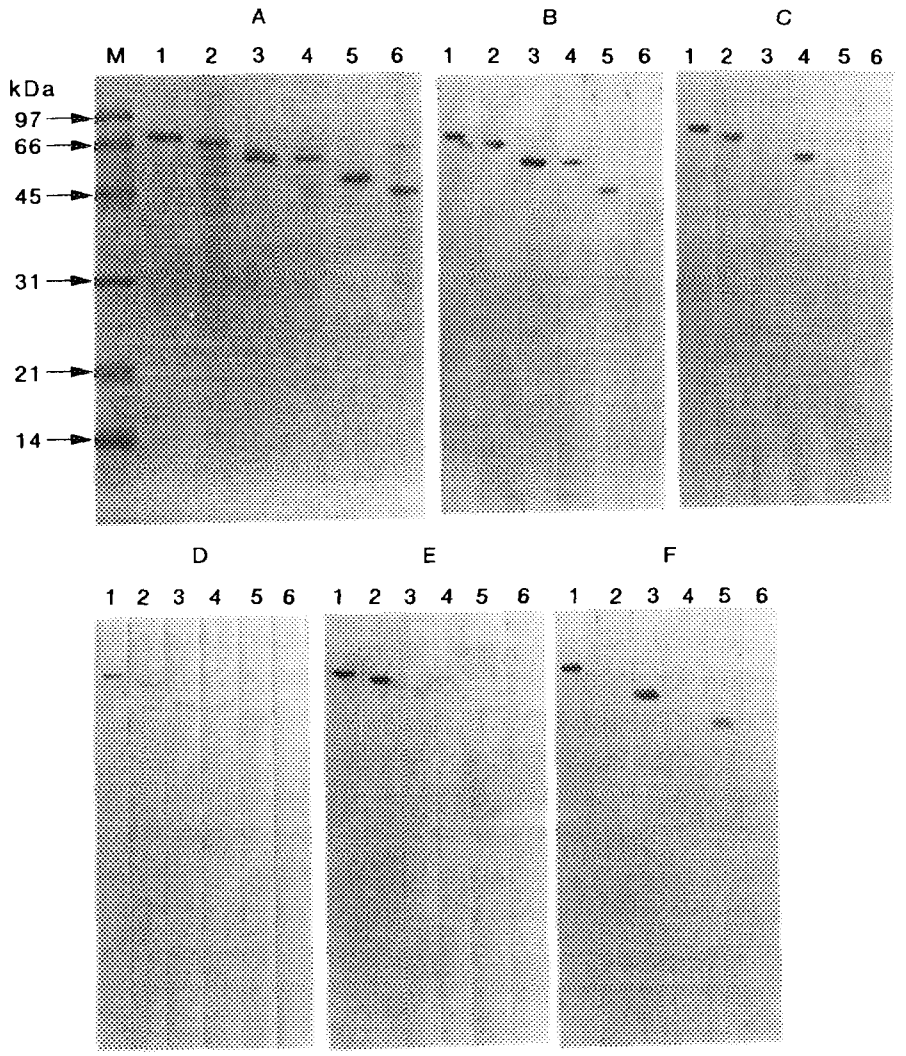


**Fig.2. Immunoblotting patterns of anti-chitinase A1 MAbs.** Intact and modified chitinase A1 were loaded on a SDS-PAGE gel. Lane 1, ChiA1; lane 2, ChiA1ΔCA; lane 3, ChiA1ΔIII; lane 4, ChiA1ΔIIICA; lane 5, ChiA1Δ2III; lane 6, ChiA1ΔD2IIICA. (A) Coomassie Brilliant Blue(CBB) staining. 5 μg of protein was applied to each lane of SDS-PAGE. M, molecular mass standards. (B)-(F) Immunostaining with MAbs G-2-5M, G-3-20M, D-31-5M, 2D-20-5M and 2F-13-2M. 0.25 μg of protein was applied to each lane.

of R-1 and R-2, consisting mostly of R-1 sequences and the C-terminal region of R-2. Since G-3-20M did not react with ChiA1Δ2III, the MAb most probably recognizes the type III region of the enzyme. Furthermore as the MAb reacted with ChiA1ΔIIICA and not with ChiA1ΔIII(the latter lacks the R-1-R-2 junction region), G-3-20M is thought to recognize the region at or near the junction of the two type III modules.

MAb D-31-5M reacted with ChiA1 and modified chitinases including ChiA1ΔCA, ChiA1ΔIII and ChiA1ΔIIICA, but not with ChiA1Δ2III and ChiA1Δ2IIICA (Fig. 2D). This MAb also recognizes the type III region as it did not react with ChiA1Δ2III. The

region recognized by this MAb is neither the junction region nor the C-terminal part of R-2, because it recognized ChiA1ΔIII and ChiA1ΔIIICA. Therefore, the MAb is thought to recognize the first type III module (R-1 type III module) or, alternatively, an epitope common to both the R-1 and R-2 type III modules. We could not distinguish these two possibilites from our data.

MAb 2D-20-5M was found to recognize R-2 as it reacted with ChiA1 and ChiA1ΔCA but not with ChiA1ΔIII, ChiA1ΔIIICA, ChiA1Δ2III or ChiA1Δ2IIICA (Fig. 2E). The region missing in ChiA1ΔIII, ChiA1ΔIIICA and ChiA1Δ2III is R-2 type III module.

The two MAbs 2B-11-5M and 2F-13-2M reacted with ChiA1, ChiA1ΔIII and ChiA1Δ2III, but not with ChiA1ΔCA, ChiA1ΔIIICA or ChiA1Δ2IIICA, indicating that the MAbs recognize the C-terminal chitin binding domain CA since modified chitinases lack C-terminal domains. Western blot results obtained with 2F-13-2M are shown in Fig. 2F.

In summary, among the eleven MAbs obtained, six recognized the catalytic domain; three, the type III region; and two, the chitin binding domain. The three MAbs recognizing type III regions include one recognizing the first type III modules, another recognizing the second type III module and the third, the region containing the junction between the two type III modules. Thus, specific antibodies recognizing each of the four discrete domains of mature form chitinase A1 were obtained.

***The effect of MAbs on the activity of chitinase A1***

The effect of the eleven MAbs on the catalytic activity of chitinase A1 were examined using *p*NP-(GlcNAc)3 as a chitinase assay substrate. Intact chitinase A1 (5 μg) and 0.05ml of hybridoma culture supernatant were mixed and chitinase activity was measured after incubating 30 min. at 25 °C. As shown in Fig. 3, none of the MAbs inhibited chitinase activity. In addition to this unexpected result, two MAbs G-2-5M and 2E-10-3M, enhanced chitinase A1 activity significantly while the other MAbs did not have any significant effect on the hydrolyzing activity toward *p*NP-(GlcNAc)3. MAb G-2-5M enhanced chitinase activity approximately 3 fold while 2E-10-3M enhanced it 1.5 fold. Under the experimental conditions used here, chitinase activity increased gradually and reached a maximum rate after 30 min. incubation. (data not shown) The activation mediated by G-2-5M was dose-dependent and maximal activation, approximately 7 fold above normal, was observed in the presence of 250 μg/ml of antibody (Fig. 4). The anti-human carcino embryonic antigen (CEA) monoclonal antibody (Chemicon International, Inc., California, USA) used as a control IgG1 antibody did not have any effect on the activity of chitinase A1 (see Fig. 4).

It is not at all clear how these MAbs activate chitinase A1. An obvious possibility would be that activation may be result from an induced conformational change in the catalytic domain due to binding by the MAbs.

***Cross-reactions of MAbs with chitinase D***

As we reported previously, the C-terminal chitin binding domain (CA) of chitinase A1 and the N-terminal domain (ND) of chitinase D have 61.7% amino acid identity (5). The type III module RD in chitinase D showed 62.8 and 60.6% amino acid matches,

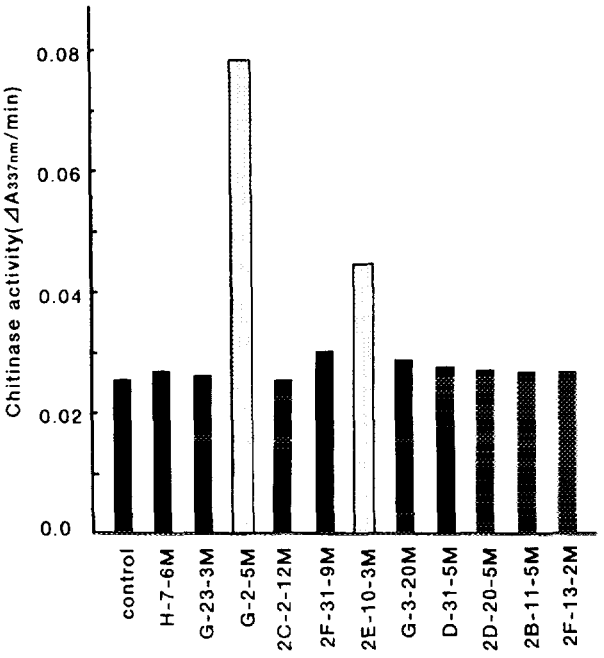


**Fig.3. Effect of MAbs on the activity of chitinase A1.** Culture supernatants (50 μl) of MAb secreting hybridoma lines and intact chitinase A1 (5 μg) were mixed, and chitinase activity was measured after incubating 30 min. at 25 °C. In the control assay, culture medium was used instead of the culture supernatant.

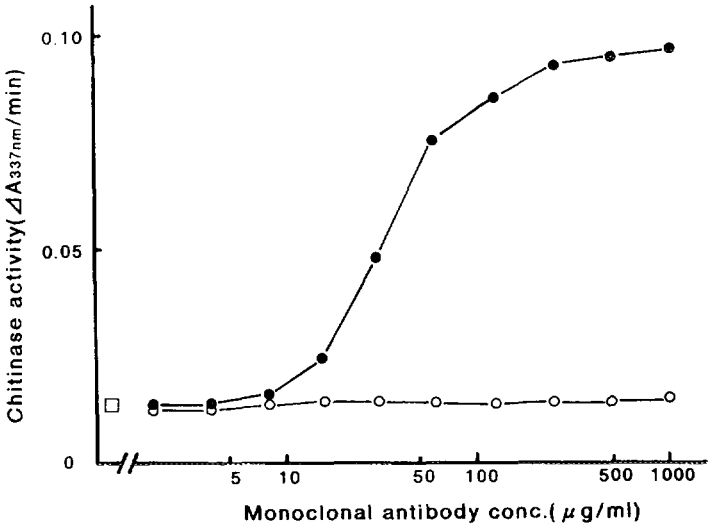


**Fig.4. Activation of chitinase A1 by different concentrations of MAb G-2-5M.** Chitinase A1 (5 μg) and various IgG concentrations of G-2-5M, which was purified as described in Materials and Methods, were mixed and chitinase activity was measured after 30 min. incubation. An anti-human CEA monoclonal antibody with same Ig subclass IgG1 as G-2-5M was used as the control MAb. ●, G-2-5M, ○, anti-human CEA antibody, □, without antibody.

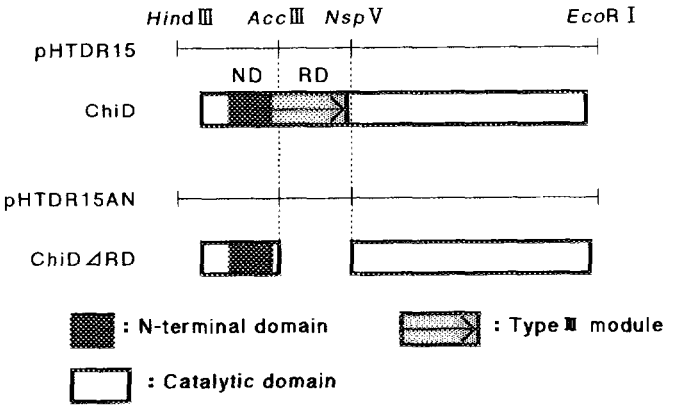


**Fig.5. Domain organization of intact and modified chitinase D molecules lacking type III module.**

respectively, to the type III modules R-1 and R-2 in chitinase A1. Such high sequence similarity suggested the possibility that MAbs recognizing type III modules or the chitin binding domain of chitinase A1 might also recognize corresponding structures in chitinase D. To test this idea the cross reactivities of G-3-20M (recognizes the region containing the junction of two type III modules), D-31-5M (recognizes R-1), 2D-20-5M (recognizes R-2), 2B-11-5M and 2F-13-2M (recognize CA) to chitinase D were examined.

Western-blotting analysis was thus carried out using purified preparations of intact chitinase A1, intact chitinase D and modified chitinase D lacking the type III module. The domain structures of chitinase D and modified chitinase D, together with the restriction map of the encoding DNA fragment, are summarizied in Fig. 5. None of the MAbs which recognized the type III region of chitinase A1 cross-reacted with either intact or modified chitinase D (Fig. 6 B-D) despite extensive amino acid sequence similarity. These MAbs were found to specifically react only with the type III of chitinase A1.

As for the two MAbs which recognized the chitin binding domain of chitinase A1, one MAb (2F-13-2M) was found to react with both intact and modified chitinase D (Fig. 6F) but the other, 2B-11-5M, did not (Fig. 6E), suggesting that the recognition sites of the two MAbs are distinct.

Thus, in spite of the high degree of similarity between the two chitinases, 2F-13-2M was the only MAb to cross-react with chitinase D among the five MAbs which recognized the type III region or the chitin binding domain of chitinase A1.

The C-terminal domain of chitinase A1 is a chitin binding domain (CA) and is required for efficient hydrolysis of chitin. Type III modules (R-1 or R-2) are not directly involved in chitin binding but play an important role in the hydrolysis of chitin by the enzyme bound to chitin (3). Detailed studies on the chitin binding domain and the type III domains are necessary to understand how chitinases degrade this insoluble and stable polysaccharide. Thus, in order to study the mechanisms and properties of chitin binding

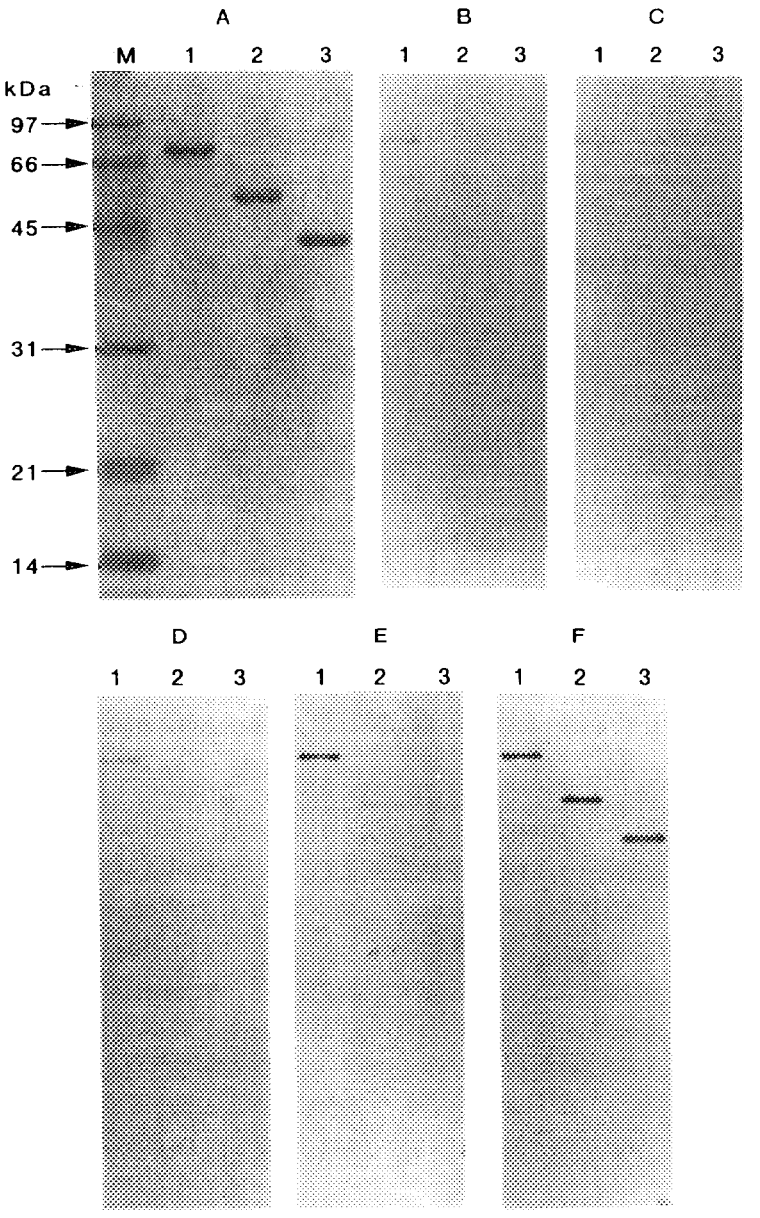


**Fig.6. Cross-reaction of MAbs with intact and modified chitinase D as detected by Western blotting.**
(A) Coomassie Brilliant Blue (CBB) staining. Lane 1, chitinase A1; lane 2, chitinase D; lane 3, modified chitinase D lacking type III module. 5 μg of protein was applied to each lane of the SDS-PAGE gel. (B)-(F)Immunostaining with G-3-20M, D-31-5M, 2D-20-5M, 2B-11-5M and 2F-13-2M. 0.25 μg of protein was applied to each lane.

as well as to elucidate the structures of the chitin binding domain and type III domain, preparation of isolated domains and their anaysis are indispensable. The MAbs specific for each domain of chitinase A1 provide powerful tools for the detection and purification of isolated domains.

The MAbs obtained here will undoubtedly be very useful in studies aimed at understanding the chitinase system of *B. circulans* WL-12. Chitinases observed in the culture supernatant of this bacterium are thought to be derived from at least four distinct chitinase genes and the proteolytic modification of the gene products result in more than six distinct chitinase molecules in the culture supernatant. The MAbs will provide a highly sensitive way to identify and quantify chitinase A1 and its degradation products observed in the culture supernatant.

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