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Note

Purification and Some Properties of Chitinase B1 from *Bacillus circulans* WL-12

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Basillus circulans WL-12 is one of the bacteria that excrete chitinases into culture media. Six major chitinases (A1, A2, B1, B2, C, and D) were detected in the culture supernatant of this bacterium, when chitin was included in the culture medium. Among these chitinases, chitinase A1 is the key enzyme in the chitinase system, and chitinase A2 is a derivative of A1. Chitinase B2 was considered to be derived from B1, since they share an identical N-terminal amino acid sequence.[1,2] Chitinase B1 (and B2) is the next most abundant enzyme in the chitinase system. Thus, the enzymatic nature and the elucidation of the function of chitinase B1 are essential for understanding the mechanism of chitin degradation by this bacterium.

As the first step in studying the role of chitinase B1, the enzyme was purified from the culture supernatant of *B. circulans* WL-12 grown in the presence of chitin. Since chitinase B2 was not detected early in the cultivation, the crude enzyme preparation from the day 1 culture supernatant of *B. circulans* WL-12 was used as a starting enzyme for chitinase B1 purification. Cultivation of the bacterium and the preparation of the crude enzyme were described in our previous report.[1] Chitinase B1 was precipitated between 40 and 60% saturation of ammonium sulfate from the crude enzyme solution, and purified by isoelectric focusing. The activity profile of the isoelectric focusing ranging from pH 5 to 9 is shown in Fig. 1. Only one major peak of chitinase activity was observed in this range. Chitinase activity corresponding to chitinase B2 was not detected at all just as we expected. The chitinase peak fractions corresponding to fraction Nos. 5 to 10 were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). As shown in Fig. 2A, the peak fractions, 7, 8, and 9, contained only chitinase B1 and no other protein band was detected. Purified chitinase B1, fractions 7, 8, and 9, did not contain other chitinase activity than chitinase B1, as shown in Fig. 2B. Recovery of the chitinase activity measured with glycol chitin as an assay substrate was 8.6%, and the increase in specific activity was 10-fold. The estimated size by SDS-PAGE and p*I* of the chitinase B1 were 38 kDa and 6.6, respectively, as described before.[1]

Optimum pH and temperature of the purified chitinase B1, measured using glycol chitin as an assay substrate, were 4.5 and 65°C, respectively. The enzyme maintained 100% activity at 60°C, 50% activity at 70°C, and 0 activity at 75°C after 10 min of incubation. The hydrolysis products from colloidal chitin generated by the action of chitinase B1 were predominantly $(GlcNAc)_2$ and a very small amount of GlcNAc. No oligosaccharides larger than $(GlcNAc)_3$ were detected during the reaction.

To compare the substrate specificities of chitinases B1 and A1, the key enzymes of the chitinase system, specific hydrolysis rates of various chitin-related compounds by purified chitinases A1 and B1 were calculated (Table I). Chitinase A1 was purified by the combination of chitin adsorption and Sephadex G-100 gel filtration chromatography as described in our previous report.[1] The reaction mixture (1.5 ml) contained 0.133% (w/v) of substrate and 2.5 μg of the enzyme. After incubation at 37°C for 10 min, the reaction was stopped, and reducing

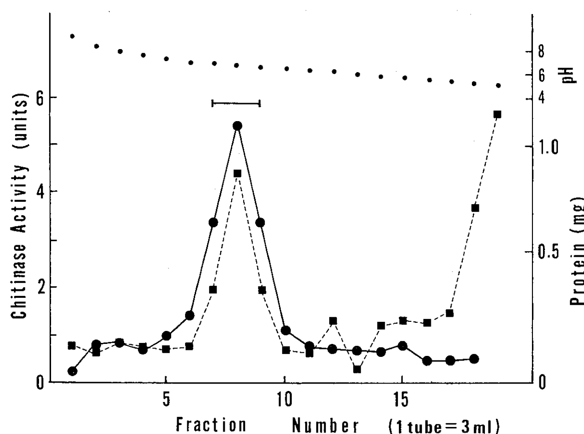

Fig. 1. Activity Profile of Isoelectric Focusing for Chitinase B1 Purification.

Symbols: ●, chitinase activity measured by the modified Schales' procedure[3] with glycol chitin as an assay substrate; ■, protein measured by the method of Lowry *et al.*[4]; ···, pH. The bar indicates the fractions which contain only chitinase B1 protein.

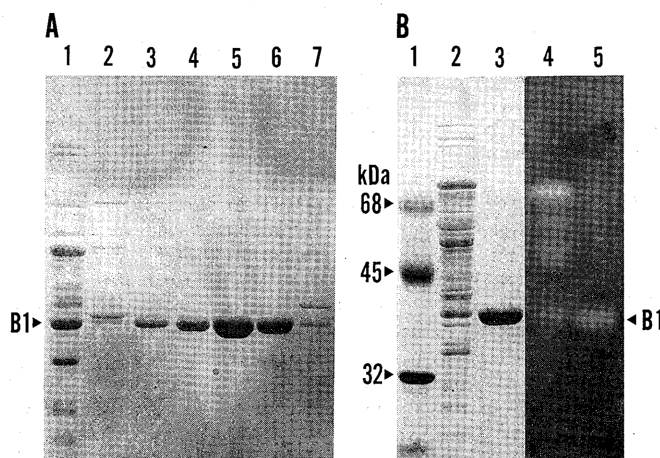

Fig. 2. SDS-PAGE Analysis of the Chitinase Peak Fractions of the Isoelectric Focusing (A) and Purified Chitinase B1 (B).

(A) Lane 1, proteins precipitated between 40 and 60% saturation of ammonium sulfate; lane 2 to 7, fractions 5 to 10 of Fig. 1. (B) Lane 1, size marker; lane 2, crude enzyme; lane 3, purified chitinase B1; lane 4, chitinase activity of the crude enzyme (lane 2) detected on agar replica of SDS-PAGE; lane 5, chitinase activity of the purified chitinase B1 (lane 3). The procedure for the detection of the chitinase activity in the polyacrylamide gel was described in our previous reports.[1,5]

Table I. Specific Hydrolysis Rates of Chitinase A1 and B1 against Several Chitin-related Compounds

Substrate[a]	Specific hydrolysis rate (moles of reducing sugar/mole protein per min) Chitinase A1	Chitinase B1
Colloidal chitin	457	59
Powdered chitin	4	ND[b]
Glycol chitin	42	441
Carboxylmethyl chitin	688	399
Chitosan	14	45

[a] Powdered chitin, carboxymethyl chitin, and chitosan (8B) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Colloidal chitin and glycol chitin were prepared by the methods of Berger and Reynolds,[6] and Yamada and Imoto,[7] respectively.

[b] Not detected.

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sugar generated during the reaction was measured by a modification of Schales' procedure[3] using GlcNAc as standard.

Chitinase A1 hydrolyzed colloidal chitin better than glycol chitin (Table I). On the other hand, the hydrolysis rate of glycol chitin by chitinase B1 was much faster than that of colloidal chitin. Both chitinases hydrolyzed carboxymethyl chitin very well. Hydrolysis of powdered chitin by chitinase B1 was not detectable under our experimental conditions. Chitinase B1 hydrolyzed chitosan (80% deacetylated) significantly. As demonstrated in this experiment, the substrate specificities of chitinase A1 and B1 are quite different. Chitinase B1 hydrolyzed soluble substrates well but not insoluble substrates. Chitinase A1 hydrolyzed insoluble substrates much more efficiently than chitinase B1 did.

Since chitinase B1 did not hydrolyze insoluble substrates efficiently, hydrolysis of soluble oligosaccharides generated by the action of the other chitinase was assumed to be the role of chitinase B1. However, all chitinases of the bacterium so far tested, including chitinase A1, generated predominantly $(GlcNAc)_2$ directly from colloidal chitin. Oligosaccharides larger than $(GlcNAc)_3$ were not detected in either case. Thus, hydrolysis of oligosaccharides generated by the action of the other chitinases is not likely to be the role of chitinase B1. In order to find out the real roles of chitinase B1 in the chitinase system of the bacterium, further detailed analyses of enzymatic properties are required.

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