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Glucanases and chitinases of *Bacillus circulans* WL-12

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SUMMARY

Lysis of cell walls of various yeast species by β-1,3- and β-1,6-glucanases of *Bacillus circulans* WL-12 was investigated. Selective enzymolysis of cell walls of *Pyricularia oryzae* by single and combined actions of β-1,3-,β-1,6-glucanases and chitinase was followed. Chemical structure of the cell wall glucan of *P. oryzae* was determined by chemical and enzymatic methods. Multiple component nature of glucanases of *B. circulans* WL-12, their induction and lytic actions on cell walls of various yeasts were studied. Genes specifying glucanases and chitinases of *B. circulans* WL-12 were cloned in *E. coli*, and their nucleotide sequences were determined. Fibronectin type III modules were found in the chitinases. Functions of the domains of the deduced structures of the glucanases and the chitinases were studied by various methods including molecular genetic techniques.

INTRODUCTION

To explore the possibility of introducing enzymatic techniques for comparative studies of the cell wall composition of different yeasts, we began by isolating microorganisms capable of lysing yeast cell walls. About 30 strains of microorganisms were isolated which produced lysed zones around colonies on plates containing cell walls of bakers' yeast. We selected a strain of bacterium which was identified as *Bacillus circulans*. The strain was designated as *B. circulans* WL-12. The strain produced a strong lytic activity of walls of *Saccharomyces cerevisiae*, and secreted β-1,3-(substrate laminarin) and β-1,6-(substrate pustulan) glucanase activities into the medium.

In this article, we begin with the description of lysis of the cell walls of many species of yeast by the single and combined activities of β-1,3- and β-1,6-glucanases, trying to correlate the results with the taxonomy of the yeasts. We then conducted selective enzymolysis of cell walls of *Pyricularia oryzae*, a rice blast fungus, as a model of filamentous fungi. Then the chemical structure of the cell wall glucan of *P. oryzae* was determined by the combination of chemical and enzymatic methods. The multiple nature of glucanases led us to investigate their induction. Modes of decomposition of cell walls of *P. oryzae* and various species of yeast by the crude enzymes obtained using the cell walls of *P. oryzae* and various species of yeast as inducer substrates were compared. We then focus on our recent genetic analysis which established that some of the multiple glucanases were generated proteolytically from a common precursor. Functions of domains of one of the glucanases are described, as is genetic analysis of the chitinases of *B. circulans* WL-12. Fibronectin type III modules were found, for the first time, in prokaryotic enzymes. Molecular genetic techniques were applied to clarify the functions of chitinase domains. This bacterium secretes a number of polysaccharide-degrading enzymes: β-1,3 and β-1,6 glucanases [24,28], β-1,3 and β-1,4-glucanases [5,14], α-1,3-glucanases [16], amylase [24], chitinase [27], and xylanase [8].

Decomposition of walls of different yeasts by endo-β-glucanases [24,28,29]

When *B. circulans* WL-12 was grown with cell walls of bakers' yeast as a sole source of carbon, β-1,3 and β-1,6 glucanases were produced in the medium. These enzymes were separated by DEAE column chromatography. They were applied separately and together to wall suspensions of different yeast species, and profound differences in sensitivity were observed.

(i) Extensive lysis by β-1,3-glucanase but weak and incomplete lysis by β-1,6 glucanase (e.g. *Saccharomyces cerevisiae*).
(ii) Extensive lysis by either of the glucanases (e.g. *Pichia (Hansenula) anomala, P. (H.) ciferrii, Nadsonia elongata, Ashbya gossypii*).
(iii) Weak lysis by either of the glucanases separately or combined (e.g. species of *Schizosaccharomyces, Lodderomyces (Saccharomyces) elongisporus*).
(iv) No action by the combined glucanases (e.g. *Rhodotorula rubra*).

Walls of *Debaryomyces hansenii* were only partially and weakly digested by the individual glucanases but were lysed extensively by the combined enzyme preparation.

We devised a technique which was referred to as the 'cross

This paper is dedicated to Professor Herman Jan Phaff in honor of his 50 years of active research which still continues.

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induction test'. *B. circulans* WL-12 was streaked in a straight line on a rectangular agar block containing the walls of species A (the inducer). Several small square test blocks of agar containing the walls of yeast species A (control), B, C, D and others were pushed against the central agar block. By observing the clearing of the central block and the action of the induced enzymes as they diffused into the square blocks, similarities and differences in the cell wall composition could be estimated. For example, this test gave evidence of significant differences in the cell wall composition of *Lodderomyces elongisporus, S. cerevisiae* and *Schizosaccharomyces* sp.

The cell walls of *Pichia anomala* were studied in considerable detail, and the results were compared to those obtained with *S. cerevisiae. P. anomala* glucan appeared to have more β-1,6-linkages than β-1,3-linkages, and each type of linkages seemed to occur in blocks of several repeating units, alternating with each other.

Decomposition of fungal walls by the single and combined action of β-1,3-glucanase, chitinase, and β-1,6-glucanase [27].

When *B. circulans* WL-12 was grown in a medium containing *Pyricularia oryzae* (a rice blast fungus) cell walls, β-1,3-glucanase, β-1,6-glucanases and chitinase activities were produced. These enzymes were separated from each other by column chromatography. Lysis of *P. oryzae* cell walls by single and combined action of these enzymes (chitinase of *Streptomyces* CH9 was used) were studied.

β-1,3-Glucanase effected a substantial decrease in the turbidity of *P. oryzae* cell walls with only a small release of reducing sugar. Turbidity of the cell wall suspension was reduced to 36% of the original value after 20 h of incubation while concomitant release of reducing sugar was only 10.7% of the cell wall material (expressed as glucose equivalents). Some reduction of turbidity and a small amount of reducing sugar liberation occurred with chitinase. With β-1,6-glucanase, a considerable amount of reducing sugar was released from the cell walls, although the concomitant decrease in turbidity was only slight. The amount of reducing sugar liberated during 20 h reached 24% of the wall material. Turbidity became 71% of the original value during this period. These results suggested that β-1,6-linkages may exist as short side branches of β-1,3-glucan. With the combined action of β-1,3-glucanase and chitinase, the initial rate of turbidity reduction was higher than the sum of the rates with the individual enzymes. In the combined action of β-1,3-glucanase and β-1,6-glucanase, turbidity reduction was similar to that of β-1,3-glucanase alone. Liberation of reducing sugar, however, was roughly equal to the sum of the amounts liberated by individual enzymes. Selective enzymolysis of *P. oryzae* cell walls by single and combined activities of β-1,3-, β-1,6-glucanases and chitinase gave evidence that β-1,3-glucosidic bonds in the glucan and chitin confer rigidity to cell walls.

We then proceeded to study the chemical structure of the cell-wall glucan of *Pyricularia oryzae* [18]. Through methylation, periodate oxidation and enzymatic treatments, we proposed a possible structure of the cell wall glucan. The proposed structure was consistent with our previous results of the selective enzymatic degradation of *P. oryzae* cell walls by the lytic enzymes of *B. circulans* WL-12. We concluded that the β-glucan backbone is mainly constituted of β-1,3-glucosidic linkages, and has many short side chains with β-1,6-glucosidic linkages.

β-1,3-glucanases of BC*WL-12*

(A) Multiple component nature of β-1,3-glucanase system [13]. *B. circulans* WL-12 produced multiple β-1,3-glucanases when grown on *P. oryzae* cell wall as an inducer substrate. We analyzed the crude enzyme by vertical polyacrylamide gel electrophoresis with a separation gel of 2.4 × 12 × 1 cm. After the electrophoretic run, the gel was cut into 1.0 ×1.2 × 12-cm pieces and sliced, starting from the cathode side, into pieces of 2-mm thickness. Enzymes were extracted with buffer, and β-1,3-glucanases (substrate laminarin) peaks were designated as F(fraction)-I; F-II, F-III and F-IV from the cathode side. F-I was further separated into three peaks, F-I_a, F-I_b and F-I_c. Two bands of chitinase activity were resolved by this electrophoresis. No β-1,6-glucanase activity was detected under this test condition. Activity profiles towards *P. oryzae* mycelium, *Saccharomyces cerevisiae* cell walls, pachyman (a β-1,3-glucan), *Phytophthora* glucan (a β-1,3-β-1,6-glucan), and oat glucan (a β-1, 3-β-1,4 glucan) were compared. Conspicuous differences in the activities towards oat glucan were detected among the separated β-1,3-glucanases. Oat glucan was actively decomposed by enzyme species F-I_a, F-I_b and F-I_c. Cell walls of *P. oryzae* were extensively decomposed by the enzymes in F-I region, and among them F-I_a was the most active.

(B) Glucanase F-I_a [14]. β-Glucanase F-I_a was purified to homogeneity to give a single protein band in disc gel electrophoresis. The molecular weight was estimated to be 48 000 by the SDS PAGE method of Weber and Osborn [39] as modified by Hayashi and Ohba [11]. Optimum pH with laminarin as a substrate was around 6.0. Laminarin was decomposed randomly, accumulating mainly glucose and laminaribiose after prolonged incubation. Oat glucan was actively hydrolyzed, and accumulated 3-*O*-β-D-cellobiosyl-D-glucose and 3-*O*-β-D-cellotriosyl-D-glucose as the major products of hydrolysis. These results indicated that F-I_a hydrolyzes either β-1,3 or β-1,4 glucosidic linkages in β-1,3-β-1,4 mixed linkage glucans (oat, barley glucans and lichenan), provided that either one of the linkages is adjacent to a β-1,3 glucosidic linkage.

(C) Concerted induction of β-glucanases in response to various yeast glucans [25]. *B. circulans* WL-12 was grown with cells of six species of yeasts as inducer substrates and the production of β-glucanases production was followed. Also, the isoelectric focusing patterns of β-1,3 and β-1,6-glucanases were determined. Conspicuous differences were observed among the isoelectric focusing patterns of the crude enzymes produced with different species as inducer substrates. Lysis of the cell walls of *P. oryzae* P_2 and several other yeast species with crude enzymes obtained with different inducer substrates

were compared. Although activity per ml of β-1,3-glucanase (laminarin as the substrate) was adjusted to approximately equal value, rates of turbidity reduction of any one of the cell wall suspensions were considerably different.

(D) Cloning of the genes encoding β-1,3-glucanases of B. circulans *WL-12 [38].* When *B. circulans* WL-12 was grown on pachyman as a sole source of carbon, six distinct active molecules of β-1,3-glucanase with different molecular weights were detected in the culture supernatant fluid. These were designated as glucanases A1, A2, A3, A4 (pI 4), B (pI 6) and C (pI 7).

Molecular cloning of one of the genes encoding β-1,3-glucanases, glucanase A1 (*glcA*) was achieved by transforming *E. coli* HB101 cells with recombinant plasmids bearing chromosomal DNA fragments from *B. circulans* WL-12 in the plasmid vector pUC19. A recombinant containing 4.4 kb of inserted DNA in the *PstI* site of pUC19, designated as pNT003, conferred the ability on *E. coli* to degrade pachyman. β-1,3-Glucanase activity detected in *E. coli* was mainly recovered in the periplasmic fraction. SDS-PAGE analysis revealed that the periplasmic fraction contained four active molecules of β-1,3-glucanase, which corresponded to four (A1, A2, and A3 and A4) of the six active molecules produced in the culture supernatant by *B. circulans* WL-12. No β-1,3-glucanase was produced with *E. coli* cells carrying pUC19.

(E) Structure of the gene encoding β-1,3-glucanase A1 of B. circulans *WL-12 [40].* The nucleotide sequence of the *glcA* gene, encoding the precursor of an extracellular β-1,3-glucanase A1, was determined. The putative *glcA* gene was 2046 bp long, specifying a polypeptide of 682 amino acids. The N-terminal amino acid sequence of glucanase produced in *E. coli* harboring the cloned *glcA* gene was identical to that of glucanase A1 prepared from the culture fluid of *B. circulans* WL-12 (Fig. 1). In both proteins, cleavage of the signal sequence of the glucanase precursor occurred between Ala-38 and Ala-39 of the predicted sequence.

(F) Domain structure of β-1,3-glucanase A1 [34]. As mentioned above, the four active species of β-1,3-glucanase detected in the periplasmic space of *E. coli* cells carrying the *glcA* gene, correspond to four (A1, A2, A3, and A4) of the six glucanases detected in the culture supernatant of *B. circulans* WL-12. We suspected that active fragments A2, A3, and A4 from both *B. circulans* WL-12 and *E. coli* harboring the *glcA* gene were generated by proteolytic actions on A1. To test this, limited proteolysis of β-1,3-glucanase A1 by three different commercially available proteases, namely, trypsin, chymotrypsin and papain was conducted. Each treatment gave three major fragments. The sizes of the three major fragments generated by each protease treatment were identical to those of A2, A3 and A4. These results suggested a four-domain structure for the enzyme (Fig.1). At the N-terminus of the glucanase, duplicated segments of approximately 100 amino acids were observed. N-terminal amino acid sequence analysis revealed that the active fragments corresponding to A2 and A3 lacked the first domain or first and second domains, respectively. The fragment corresponding to A4 lacked both duplicated segments, as well as the following *ca* 120-amino acid region. In losing the first, second, and third domains, the glucanase progressively lost the ability to bind to pachyman. The loss of these binding domains remarkably decreased pachyman-hydrolyzing activity, indicating that the binding activity is essential for the efficient hydrolysis of insoluble β-1,3-glucan.

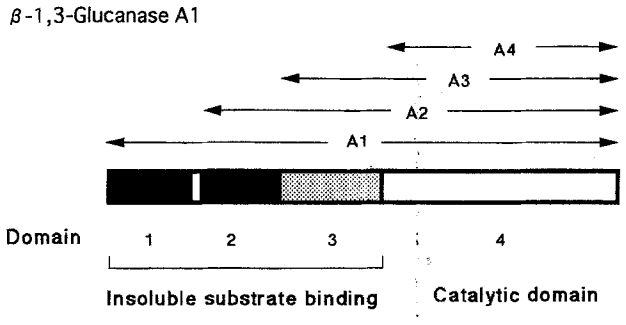

Fig. 1. Schematic representation of glucanase A1 of *B. circulans* WL-12. A1, A2, A3 and A4 represent glucanases A1, A2, A3 and A4.

(G) β-1,3-Glucanase B [19]. N-terminal amino acid sequence analysis of purified glucanases B and C suggested that C was generated from B by proteolytic modification. Chromosomal DNA prepared from *B. circulans* WL-12 was partially digested by *TaqI*, DNA fragments (4–10 kb) were collected and inserted into the *AccI* site of pUC19. Periplasmic proteins from one of the transformants of *E. coli* JM109 showing clear zones on pachyman plates contained active glucanases corresponding to B and C on SDS-PAGE. N-terminal amino acids sequence of these glucanases coincided with those of B and C produced by *B. circulans* WL-12. A 3.8-kb DNA fragment in a recombinant plasmid, pMTS21, contained the putative *glcB* gene. DNA sequence analysis revealed one open reading frame of 1239 bp which may specify the precursor of glucanase B. The N-terminus of glucanase B purified coincided with the 29th residue (Ala) of the deduced precursor base sequence. The sequence of the first 28 amino acids showed the general characteristics of prokaryotic signal peptide. The mature glucanase B deduced from DNA sequence contains 384 amino acids, and the calculated molecular size of 42 280 Da agrees with the value estimated by SDS-PAGE for glucanase B. The N-terminal region (about 240 amino acids) showed amino acid sequence similarities with the putative catalytic site regions of glucanase A1 of *B. circulans* WL-12 [40], and β-1,3-1,4-glucan 4-glucanohydrolases of *Bacillus* spp. [3,17]. The C-terminal region contained three repeating sequences of 40–55 amino acids. This region shows amino acid sequence similarities with the substrate binding domains of the yeast lytic protease I of *Rarobacter faecitabidus* [23], xylanase A of *Streptomyces lividans* [21] and β-1,3-glucanase of *Oerskovia xanthineolytica* [22].

Glucanase B showed 3- to 4-fold higher specific activities towards β-1,3-1,4 mixed linkage glucans, (e.g. oat glucan and lichenan), than towards laminarin (a β-1,3-glucan). On the other hand, glucanase A1 of *B. circulans* WL-12 showed no

activity towards β-1,3-1,4-mixed linkage glucans [1,19]. Thus, by many measures, glucanase F-I_a from *B. circulans* WL-12 resembles glucanase B, and they are probably the same enzyme.

To conclude the review of our work on glucanases of *B. circulans* WL-12, and proceed to the review of our work on the chitinases of the organism, we have to mention that research on the multiple glucanases of *B. circulans* WL-12 has also been published by a number of other investigators [6,7,10,20].

Chitinases of BC*WL-12*

(A) Cloning and sequencing of the gene encoding chitinase A1 of B. circulans *WL-12 [36,37].* When *B. circulans* WL-12 was grown in medium containing chitin as a sole source of carbon, at least six distinct chitinase species were detected in the culture supernatant. They were designated as chitinase A1, A2, B1, B2, C and D.

The *chiA* gene encoding chitinase A1 was cloned into *E. coli* by transforming HB 101 cells with recombinant plasmid vector pKK223-3 (an expression vector [4]). DNA sequence analysis revealed one open reading frame of 2097 bp which presumably corresponds to the precursor of chitinase A1. The precursor of chitinase A1 contained a long signal sequence of 41 amino acids, with an extremely long N terminal hydrophilic segment of 15 amino acids. N-terminal amino acid sequence analysis revealed that the cloned chitinase enzyme produced in *E. coli* had, at its N-terminus, an additional 8 amino acids that were not found in *B. circulans* WL-12 mature chitinase A1. The N-terminal two-thirds of the deduced amino acid sequence of chitinase A1 showed a 33% amino acid match to chitinase A of *Serratia marcescens*. Prokaryotic chitinases, class III plant chitinases, yeast chitinases, and endo-β-*N*-acetylglucosaminidases share weak amino acid sequence similarities at certain regions of each enzyme [35,37]. These regions have been assumed to be important for catalytic activities of the enzymes.

(B) Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 as essential residues for chitinase activity [31]. Based on these assignments of possible catalytic regions, three amino acid residues (Ser-160, Asp-200, Glu-204) in the cloned chitinase A1 gene of *B. circulans* WL-12 were chosen, and altered by site-directed mutagenesis. Kinetic parameters (k_{cat} and *Km*) for 4-methylumbelliferyl-*N*, *N'*, *N''*-triacetylchitotriose hydrolysis were determined with wild type and seven mutant chitinases. Chitinases with Glu-204 → Glu mutation and Glu-204 → Asp were essentially inactive and k_{cat} values of these chitinases were approximately 1/5000 and 1/17 000 that of wild type chitinase, respectively. The Asp-200 → Asn mutation decreased the k_{cat} value to approximately 1/350 of that of the wild-type enzyme. On the other hand, the k_{cat} value was not affected by Asp-200 → Glu mutation. Thus, it appeared that Glu-204 and Asp-200 were directly involved in the catalytic events of chitinase A1. The role of the carboxyl group of Asp-200 could be fully substituted by that of a Glu residue.

(C) Presence of fibronectin type III sequences (type III motif) in chitinase A1 [37]. The putative catalytic domain of chitinase A1 was immediately followed by tandemly repeating 95-amino acid segments that were 70% homologous to each other. Statistical analysis revealed that the repeating segments were related to the type III homology units of fibronectin, a multifunctional extracellular matrix and plasma protein of higher eukaryotes.

Since we reported the occurrence of the type III motif in *B. circulans* WL-12 chitinase, the motif has been noted in a number of bacterial enzymes, especially in hydrolytic enzymes. From statistical analysis [2], it was proposed that the occurrence in bacterial extracellular carbohydrate-splitting enzymes was the result of gene acquisition from a eukaryote by horizontal transfer, long after the divergence of eukaryotes and prokaryotes.

(D) Domain structure of chitinase A1 [30,32,37]. Based on sequence features and similarities with other proteins, the precursor of chitinase A1 can be divided into four domains, namely, a signal sequence, a catalytic domain, 95-amino acid tandem repeats that have relatedness to the type III homology unit of fibronectin, and a short C-terminal segment.

In order to study, the roles of domains in chitinase A1 in chitin degradation, various deletion derivatives of chitinase A1 coded by the modified *chiA* genes were prepared. This study revealed that the C-terminal domain was essential for the specific binding of the enzyme to chitin (Fig. 2). The type III domain was not directly involved in chitin binding, but the loss of this domain decreased hydrolyzing activity for insoluble substrates. Modified chitinase lacking both the C-terminal domain and all type III domains still retained enzymatic activity.

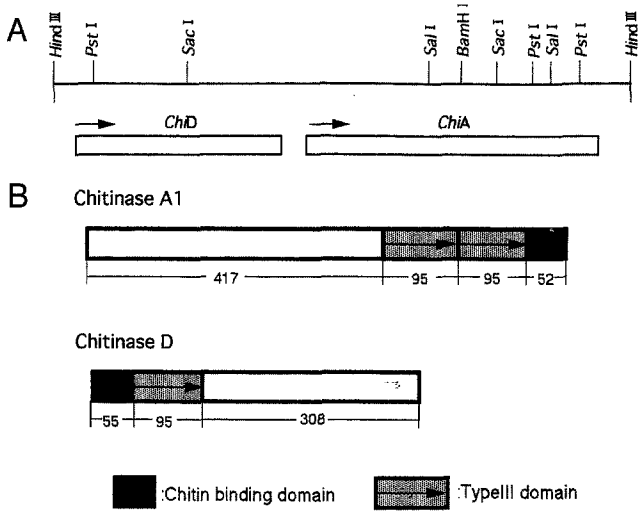

Fig. 2. Schematic representation of chitinase A1 and chitinase D of *B. circulans* WL-12. The upper diagram (A) shows the restriction map of the *Hind*III inserted DNA fragment in the plasmid pH002 carrying *chiA* and *chiD*. The lower diagram (B) shows regions of *chiA* and *chiD*. Note that *chiD* lies immediately upstream of *chiA*.

(E) Cloning and base sequence determination of the chiD *gene encoding chitinase D [35].* The nucleotide sequence of a DNA fragment specifying chitinase D was determined as well as the N-terminal amino acid sequence. It was revealed that the gene (*chiD*) encoding the precursor of chitinase D was located immediately upstream of the *chiA* gene. The deduced polypeptide specified by the *chiD* gene was 488 amino acids long, and the distance between the coding regions of the *chiA* and *chiD* genes was 103 bp. Remarkable similarity was observed between the N-terminal one-third of chitinase D and the C-terminal one-third of chitinase A1. The N-terminal 47-amino acid segment (named ND) of chitinase D showed a 61.7% amino acid match with the C-terminal segment (CA) of chitinase A1. The following 95-amino acid segment (RD) of chitinase D showed 62.8% and 60.6% amino acid matches, respectively, to the type III repeating units R-1 and R-2 in chitinase A1. A 73-amino acid segment (residues 247 to 319), located in the putative activity domain of chitinase D, showed considerable sequence similarity not only to other bacterial chitinases and class III higher plant chitinases but also to *Streptomyces plicatus* endo-β-*N*-acetylglucosaminidase H and the *Kluyveromyces lactis* killer toxin α subunit (Fig. 2(A and B)).

CONCLUDING REMARKS

In this article, research on the glucanases and chitinases of *B. circulans* WL-12 are reviewed. Lytic enzymes of *B. circulans* WL-12 have been successfully used for the preparation of fungal protoplasts [26], for the assay of β-glucan synthase [15], for the reduction of the minimal inhibitory concentrations of fungicides [33] and for the isolation of astaxanthin from *Phaffia rhodozyma* [9,12].

We conclude with a quote from The Yeasts, Vol. 4, 'Glucanases of *Bacillus circulans* WL-12 have been best studied and are becoming increasingly popular as analytical tools' [9]. Further genetic and enzymological studies on glucanases and chitinases of *B. circulans* WL-12 will give important information on bacterial hydrolytic enzymes.

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