Synthesis and Regulation of *Bacillus circulans* WL-12 1,3- β -D-Glucanases

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Bacillus circulans WL-12 1,3- β -D-glucanases are extracellular enzymes subject to catabolite repression by glucose and synthesized after the depletion of this sugar. Utilization of other complex carbon sources (1,3- β -D-glucan from baker's yeast, laminarin or xylan) resulted in a 3- to 4-fold increase in the formation of these enzymes, suggesting that they are derepressible and inducible. Under induction conditions four different enzymes were detected by isoelectric focusing that were numbered I, II, III and IV, according to their isoelectric points (3.7, 4.6, 5.5 and 6.5 respectively). Glucanase II was inducible whereas I, III and IV were both derepressible and inducible. In addition, the synthesis of glucanase II was blocked by cyclic AMP. The four enzyme forms displayed an endo- attack on laminarin and yielded similar products, but differed in some physico-chemical parameters such as molecular weight, K_m and lytic activity.

INTRODUCTION

Bacillus circulans WL-12 was isolated by Tanaka & Phaff (1965) from soil samples and characterized by its ability to lyse a number of fungal and yeast cell walls. When the micro-organism was grown with complex carbon sources, the supernatant broths contained a variety of hydrolytic enzymes such as 1,3- β -D-glucanase (Kobayashi *et al.*, 1974; Tanaka *et al.*, 1974, 1978, 1981; Fleet & Phaff, 1974; Rombouts & Phaff, 1976*b*), 1,6- β -D-glucanase (Rombouts & Phaff, 1976*a*; Johnson *et al.*, 1979), 1,3- α -D-glucanase (Johnson *et al.*, 1979; Meyer & Phaff, 1980), chitinase (Johnson *et al.*, 1979) and β -xylanase (Esteban *et al.*, 1982).

Tanaka *et al.* (1974) proposed the term 'concerted induction' to explain the formation of different forms of 1,3- β -D-glucanase when the micro-organism was grown with fungal cell walls as the carbon source. Fleet & Phaff (1974) and Johnson *et al.* (1979) considered that both the 1,3- β -D- and 1,6- β -D-glucanases were strictly inducible. In the present work we describe the induction and derepression of these enzymes (mainly 1,3- β -D-glucanases), different forms of which are present in culture supernatants depending on the carbon source used and also on the presence of exogenous cyclic AMP. We also summarize the main physico-chemical properties of glucanases I, II, III and IV (separated by isoelectric focusing) and discuss their homology with the forms identified in previous papers (Fleet & Phaff, 1974; Rombouts & Phaff, 1976*b*).

METHODS

Micro-organism and culture conditions. *Bacillus circulans* WL-12 was maintained and cultured as previously described (Esteban *et al.*, 1982). The growth medium contained yeast nitrogen base (0.7%; Difco), and carbon sources (glucose, 1,3- β -D-glucan from baker's yeast and β -xylan) at a final concentration of 0.5%. Occasionally, glucose was used at 2% (w/v). The bacterium was grown aerobically (300 ml; 1000 ml flasks) from an inoculum of 10^8 cells ml⁻¹ (3 ml; 10^7 cells ml⁻¹ final concentration) at 30 °C in a rotary shaker at 250 r.p.m. for 3 d. Glucose utilization was monitored by estimating unused glucose by the modification of Bruss & Black (1978) of Keston's method (1956).

Preparation of enzyme samples. Samples of culture were withdrawn (every 3 h and up to 68 h) and bacterial cells collected by centrifugation at 2000 *g* for 10 min. The supernatants were dialysed for 24 h at 4 °C against twice-

distilled water and total 1,3- β -D- and 1,6- β -D-glucanases determined. When higher enzyme activity per unit volume was needed, the dialysates were concentrated by ultrafiltration through Amicon PM-10 membranes. The volume of the samples could be reduced about 20-fold by this procedure with no apparent loss of enzyme activity.

Enzyme assays. 1,3- β -D- and 1,6- β -D-glucanases were assayed with laminarin and pustulan (prepared in 0.1 M-sodium acetate buffer, pH 5.2; 0.5% final concentration) as substrates, respectively. To 0.1 ml enzyme solution, 0.1 ml substrate was added and the mixture incubated at 30 °C for appropriate periods of time. The reducing power released from the substrates was determined (Somogyi, 1952; Ashwell, 1957), using glucose as the standard. One enzyme unit was defined as the amount of enzyme that catalysed the release from the substrate of 1 nmol glucose, or equivalent reducing power, per min at 30 °C. Specific activity was determined by referring the number of enzyme units either to the number of bacterial cells or to mg protein as determined by the Lowry method with bovine serum albumin as the standard.

Isoelectric focusing. Fractionation of the β -glucanases in culture broths was accomplished in a 110 ml LKB isoelectric focusing column. The density gradient was formed with glycerol, and ampholite (pH 4–6.5) was used at a concentration of 1% (w/v). The rest of the procedure was as previously described (Esteban *et al.*, 1982).

Paper chromatography. Paper chromatography was done on Whatman no. 1 paper with ethyl acetate/pyridine/water (8:2:1, by vol.) as solvent. The spots were visualized according to the method of Trevelyan *et al.* (1950).

Gel exclusion chromatography. Molecular weight determinations were done in a column (60 $\times$ 0.6 cm) packed with Biogel P-100, equilibrated with 0.1 M-sodium acetate buffer (pH 5.2) and calibrated with proteins of known molecular weights (bovine serum albumin, 67000; egg albumin, 43000; chymotrypsinogen A, 25000 and ribonuclease A, 13700). Fractions of 0.5 ml were collected and the void volume of the column was determined by the elution of blue dextran 2000.

Lytic activity. Lytic activity of the different 1,3- β -D- and 1,6- β -D-glucanases was followed by observing clearing zones in agar plates containing suspended cell walls of *Saccharomyces cerevisiae*. The method was similar to that described by Rombouts & Phaff (1976a, b).

Chemicals. Xylan (from larch wood) and cyclic AMP were obtained from Sigma, purified xylan was prepared as described previously (Esteban *et al.*, 1982) and alkali-insoluble 1,3- β -D-glucan from baker's yeast was prepared according to the procedure of Manners *et al.* (1973). Laminarin was purchased from Koch-Light Laboratories, and pustulan from Calbiochem-Behring Corp. Other chemicals were of the best quality available.

RESULTS AND DISCUSSION

Effect of different carbon sources on the production of 1,3- β -D- and 1,6- β -D-glucanases by B. circulans WL-12

Previous authors have suggested (Fleet & Phaff, 1974; Johnson *et al.*, 1979), in an apparently unambiguous way, a strictly inducible nature for *B. circulans* WL-12 1,3- β -D- and 1,6- β -D-glucanases. However, our results indicated that once glucose was depleted from the culture medium, the enzymes were synthesized; if glucose was not exhausted (i.e. growth in the presence of 2% glucose) they were not synthesized. It follows that 1,3- β -D- and 1,6- β -D-glucanases of *B. circulans* WL-12 are subject to catabolite repression and in this sense they may be considered as derepressible enzymes. Table 1 summarizes the total enzyme activities obtained by growing the bacterium with different carbon sources. With 0.5% glucose as the carbon source, the glucanase activities were about 30% of those obtained with 1,3- β -D-glucan. Xylan, even after purification (Esteban *et al.*, 1982) gave enzyme activities greater than those obtained with glucose. It is therefore likely that *B. circulans* WL-12 β -glucanases are inducible, though it is not clear why the bacterium should synthesize β -glucanases when a xylan is used as the carbon source. One explanation could be that the expression of the respective genes is in some way coupled.

Separation of 1,3- β -D-glucanases by isoelectric focusing

Tanaka *et al.* (1974, 1978, 1981) have reported that *B. circulans* WL-12 is capable of producing different β -glucanases, depending on the substrate used as the inducer. Because their studies used very complex carbon sources (whole cell walls isolated from a variety of fungi and yeasts), we thought that the use of simple substrates might shed some light on the problem of the variety of enzyme forms. Isoelectric focusing of samples from culture broths with 1,3- β -D-glucan from baker's yeast and β -xylan as carbon sources resolved four 1,3- β -D-glucanases, which we numbered I (pI 3.7), II (pI 4.6), III (pI 5.5) and IV (pI 6.5), and only one 1,6- β -D-glucanase (pI 8.1) (Fig. 1b, c). In the case of growth on 0.5% glucose, only three enzymes were detected,

Table 1. 1,3- β -D- and 1,6- β -D-glucanase activities present in the culture broths of *B. circulans* WL-12 grown with different carbon sources

The numbers represent percentages of activity as compared to those obtained with 1,3- β -D-glucan as the carbon source (100% represents 180 units ml⁻¹ for 1,3- β -D-glucanases and 105 units ml⁻¹ for 1,6- β -D-glucanases). The samples were withdrawn after 65 h growth.

Growth substrate	1,3- β -D-Glucanase	1,6- β -D-Glucanase
1,3- β -D-Glucan*	100	100
Pure xylan	59	60
Commercial xylant†	66	74
Laminarin	50	53
Glucose (0.5%)	27	33
Glucose (2%)	0	0

* From baker's yeast.

† Contaminated with callose.

Fig. 1. Isoelectric focusing of supernatant broths of *B. circulans* WL-12 grown with 0.5% glucose (a), 1,3- β -D-glucan from baker's yeast (b), purified xylan (c) and 1,3- β -D-glucan from baker's yeast supplemented with 8 mM-cyclic AMP (d). ○, 1,3- β -D-Glucanase; ●, 1,6- β -D-glucanase; ---, pH.

corresponding to forms I, III and IV (Fig. 1a), indicating that form II seems to be the only one that is strictly inducible, whereas the other three forms are derepressible.

We have previously reported (Esteban *et al.*, 1984) that cyclic AMP may control the synthesis of these enzymes as a negative effector. With this in mind we tried to find which of the enzyme forms was affected by the nucleotide when added exogenously (8 mM) to a culture growing with

Table 2. Percentages of the four forms of 1,3- β -D-glucanase obtained by growth of *B. circulans* WL-12 with different carbon sources

Data were calculated in each case from the results obtained by isoelectric focusing of the enzyme extracts (see Fig. 1) after collecting the gradient fractions which corresponded to the different isoelectric points.

1,3- β -D-Glucanase	Growth substrate:	
	1,3- β -D-Glucan	Purified xylan
I	17	17
II	20	20
III	28	24
IV	35	39

Table 3. Comparison of the main physico-chemical properties of 1,3- β -D-glucanases secreted by *B. circulans* WL-12

Property	1,3- β -D-Glucanase:			
	I	II	III	IV
Nature	Derepressible and inducible	Inducible	Derepressible and inducible	Derepressible and inducible
Mol. wt*	27000	21000	35000	18000
pI	3.7	4.6	5.5	6.5
K_m (mg ml ⁻¹)†	2.0	1.23	0.48	1.12
Hydrolysis pattern‡	Endo-acting	Endo-acting	Endo-acting	Endo-acting
Lytic activity§	-	+	+++	+

* Determined by filtration through a Biogel P-100 column calibrated with proteins of known molecular weight.

† The K_m data correspond to values obtained by using the Hill representation. The different 1,3- β -D-glucanases were incubated with increasing amounts of the substrate (laminarin), and the results obtained were used for this representation.

‡ Established by analysing hydrolysis products on paper chromatography (Whatman no. 1). Enzyme samples were incubated with the substrate (laminarin, 0.5%, w/v) at 30 °C. At time intervals, 20 μ l samples of the mixtures were withdrawn and analysed by paper chromatography after heating the samples. Spots revealed mainly glucose, laminaribiose, laminaritriose and laminaritetraose, and minor quantities of larger oligosaccharides.

§ Followed as described in Methods. -, No lytic activity; +, lytic activity, but without total clearing of the agar; + + +, total hydrolysis of the *S. cerevisiae* walls used in the assay.

1,3- β -D-glucan from baker's yeast as the carbon source. The results (Fig. 1d) revealed that form II was missing, and that cyclic AMP was abolishing the stimulatory effect of the polysaccharide. This cyclic nucleotide did not alter the secretion of these enzymes, since cell-free extracts showed no particular enzyme accumulation.

From the results shown in Fig. 1, we calculated the percentage contribution of each β -glucanase to the total activity; the values are summarized in Table 2. The four enzyme forms were synthesized in nearly the same proportion regardless of the polysaccharide used. As indicated above, the total 1,3- β -D-glucanase activity with 0.5% glucose was about 30% of that obtained with a 1,3- β -D-glucan. If we assume that only 20% is strictly inducible (form II) it would appear that polysaccharides such as 1,3- β -D-glucan from yeast and β -xylan in some way stimulate the synthesis of the remaining forms (I, III and IV).

The properties of the separated enzyme forms (Fig. 1) were studied in more detail (Table 3). Analysis of the lytic power (2 units of each enzyme form) revealed that enzyme I lacked this ability whereas the other three were each able to lyse *S. cerevisiae* cell walls, 1,3- β -D-glucanase III being the most active. Fleet & Phaff (1974) also described the existence of a non-lytic β -glucanase that could be identified with our 1,3- β -D-glucanase I, with the exception of the K_m value (0.55 mg ml⁻¹ vs 2.0 mg ml⁻¹, Table 3). With respect to the lytic 1,3- β -D-glucanases, Rombouts & Phaff (1976b) described two forms (I and II). Their 1,3- β -D-glucanase I exhibited a higher lytic power and in this sense it could be identified with the 1,3- β -D-glucanase III

described in this paper. Both have nearly the same molecular weight but again they differ somewhat in their K_m values. Tanaka *et al.* (1974) reported a complex induction pattern for *B. circulans* WL-12 1,3- β -D-glucanases using *Pyricularia oryzae* mycelium and Tanaka *et al.* (1978) described the induction of four 1,3- β -D-glucanases, when the bacterium was grown on the yeast *Debaryomyces hansenii*, that exhibited isoelectric points similar to those reported here and therefore they may be identified with our forms I, II, III and IV. In view of all these results, it is difficult to understand how some of the complex carbon sources (e.g. *Candida parapsilopsis* walls) employed by Tanaka *et al.* (1978) induced only one form of 1,3- β -D-glucanase (pI 4.2). A possible explanation may be that such walls contain a compound that in some way prevented the formation of the other 1,3- β -D-glucanases.

Bacillus circulans WL-12 produced a single 1,6- β -D-glucanase (Fig. 1). Attempts were made, unsuccessfully, to resolve this fraction (pI 8.1) by different methods, including gel permeation chromatography (over Sephacryl S-200), DEAE-Biogel ion-exchange chromatography and ultracentrifugation in a sucrose gradient.

It may be concluded from the findings reported here that *B. circulans* WL-12 produced one 1,6- β -D-glucanase and up to four 1,3- β -D-glucanases, when grown with 1,3- β -D-glucan, xylan, laminarin or 0.5% glucose as carbon source. Of these latter enzymes, one was inducible (form II), whereas the remaining ones were derepressible and inducible.

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