EFFECT OF PAPULACANDIN B AND ACULEACIN A ON β-(1,3) GLUCAN-SYNTHASE FROM *GEOTRICHUM LACTIS*

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1. Introduction

Aculeacin A is the main component of a series of antibiotics produced by *Aspergillus aculeatus* [1] and papulacandin B is produced by *Papularia sphaerosperma* [2]. These compounds inhibit the growth of several yeasts but have little or no activity against bacteria or protozoa [1,2].

Aculeacin A and papulacandin B do not cause membrane damage and ion leakage [3,4], as in the case of polyene antibiotics [5], but interfere with cell-wall synthesis in yeast. Glucose incorporation into the alkali-insoluble glucan fraction of the cell wall of *Saccharomyces cerevisiae* is selectively inhibited by those antibiotics [4]. Since glucan is the main structural component of yeast cell wall [6], the weakening of the wall would be the cause of the lysis of the cells by osmotic pressure. The possibility of measuring β-(1,3) glucan-synthase 'in vitro' in the fungus *Geotrichum lactis* has allowed us to demonstrate that papulacandin B and aculeacin A are indeed inhibitors of the synthase. We show that the inhibition is specific and occurs not only 'in vitro' but also 'in vivo' since the addition of the antibiotics to a culture leads to cell-free extracts with a partially inactive synthase. Some characteristics of the inhibition are described.

2. Materials and methods

Papulacandin B and aculeacin A were generous gifts of Dr K. Scheibli (Ciba-Geigy, Basel) and Dr K. Mizuno (Toyo Jozo, Tokyo), respectively. UDP-[U-^{14}C]glucose (240 mCi/mmol, UDP-*N*-acetyl [U-^{14}C]-glucosamine (346 mCi/mmol) and GDP-[U-^{14}C]mannose (179 mCi/mmol) were purchased from the Radiochemical Centre. UDP-glucose, UDP-*N*-acetylglucosamine, GDP-mannose and GTP were from Sigma.

2.1. *Organism and growth conditions*

Geotrichum lactis CECT 1102, whose perfect state has been described as *Endomyces geotrichum* [7] was the organism used. It was grown at 30°C in an incubator shaker in YED medium (1% glucose and 1% yeast extract).

2.2. *Enzyme preparation*

Cell-free extracts were obtained by mechanical breakage using a Braun homogenizer and glass beads. The mixture contained 7 g material (wet wt), 21 ml 1 mM EDTA (pH 7), 14 g glass beads, and was homogenized for 45 s, twice, under dry ice refrigeration. The extracts were diluted with 10 ml 1 mM EDTA and centrifuged at 750 × *g* for 5 min to remove the cell walls. The resulting supernatants were centrifuged at 48 000 × *g* for 30 min, and the pellets washed once with 25 ml 50 mM Tris–HCl (pH 7.5) containing 1 mM EDTA and 1 mM mercaptoethanol. The final pellets were resuspended in the same buffered solution containing 33% glycerol. All operations were done at 0–4°C. Chitin and mannan-synthases were measured in extracts prepared as for glucan-synthase, except that the solutions used for washing and final resuspension were 25 mM Tris–HCl (pH 7.5) containing 5 mM $MgSO_4$ and 25 mM imidazole–HCl (pH 6.5) containing 5 mM $MnCl_2$, respectively.

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2.3. *Assays*

The standard incubation mixture for glucan-synthase was as in [8] except that it always contained UDP-[^{14}C]glucose (1.5 X 10^5 cpm/μmol), 80 μM GTP, 2.5 mM EDTA and ~150 μg enzyme protein. Incubation was for 40 min at 30°C. Under these conditions the rate of the reaction was linear. The radioactivity was determined with a Beckman LS-8100 scintillation spectrometer.

Chitin and mannan-synthase activities were assayed as in [9,10]. Protein was measured according to [11]. One unit of enzyme is defined as the amount that catalyzes the incorporation of 1 μmol glucose/min, into glucan, at 30°C.

3. Results

3.1. *Inhibition of glucan-synthase*

Cell-free extracts from *G. lactis* were incubated under standard assay conditions in the presence and absence of papulacandin B and aculeacin A. Methanol was added to the controls in a similar amount to that resulting from addition of the antibiotics. Under the conditions of the experiment (table 1) 70% of the glucan-synthase activity resulted inhibited by papulacandin B and 65% by aculeacin A. Glucan-synthase activity from stationary-phase extracts, however, is not inhibited by the antibiotics. The degradation by β-(1,3) glucanases and its resistence to periodate oxidation (not shown) proved that the reaction product was in all cases a linear β-(1,3) glucan.

These antibiotics not only inhibit glucan-synthase 'in vitro' but when added to the growth medium lead to cell-free extracts with a partially inactive synthase. In a typical experiment, cultures of *G. lactis* (1ml/mg dry wt) were supplemented with 6 μg papulacandin B/ml or 18 μg aculeacin A/ml and after 90 min, the mycelium was harvested and cell-free extracts prepared. A substancial inhibition of glucan-synthase, in comparison with a control, was observed (table 1). The growth of *G. lactis* under these conditions, as well as RNA and protein synthesis, were not altered.

3.2. *Specificity of the inhibition*

The main polysaccharide components of *G. lactis* cell wall are galactomannan, glucan and chitin [12, 13]. Under conditions where glucan-synthase is inhibited (using an excess of antibiotics) both chitin and mannan-synthases are unaffected or slightly activated (table 1). These synthases appear to be localized on the plasma membrane in *S. cerevisiae* [8,14,15] and in other fungi, so it seems reasonable to conclude that glucan-synthase is specifically inhibited by papulacandin B and aculeacin A.

The lytic effect triggered by the antibiotics 'in vivo' does not seem to be caused primarily by the activation of endogenous β-glucanases because the β-(1,3) glucanase activity, as measured in cell-free extracts, was unaffected by the presence of the antibiotics (not shown). Furthermore, experiments with *Schizosaccharomyces pombe* have shown that β-(1,3) glucanase activity from cultures treated with papulacandin B is similar to that from untreated cultures.

Table 1
Effect of papulacandin B and aculeacin A on several cell wall synthases from *G. lactis* (milliunits/mg protein)

	In vitro[a]			In vivo[b]		
	Control	Papulacandin B	Aculeacin A	Control	Papulacandin B	Aculeacin A
Glucan-synthase	3.14	0.98	1.10	2.74	0.52	1.64
Chitin-synthase	13.30	13.75	14.70	12.70	13.55	14.55
Mannan-synthase	0.48	0.57	0.56	0.48	0.54	0.57

[a] Papulacandin B, 26 μg/ml, and 66 μg aculeacin A/ml, (final concentrations in the assays)
[b] *G. lactis* cultures (1 ml/mg dry wt) were treated with papulacandin B (6 μg/ml) or aculeacin A (18 μg/ml). After 90 min the mycelium was harvested and the synthase activities measured in the corresponding particulate fraction

3.3. *Characteristics of the inhibition*

Fig.1A represents Lineweaver-Burk plots of the synthase in the presence of papulacandin B. As it is apparent from the graph, both maximal velocity and K_m are modified by the addition of the antibiotic. This is a situation of mixed-type inhibition [16] in which the affinity for the substrate is increased by the addition of the inhibitor. The kinetics for aculeacin A inhibition is quite similar (fig.1B).

Another characteristic of the inhibition caused by these antibiotics is its incompleteness. Addition of increasing amounts of papulacandin B and aculeacin A, respectively, did not result in a complete inhibition of the activity. The concentrations for 50% inhibition were 1.2 μM for papulacandin B and 60 μM for aculeacin A. The inhibition is independent of the time of incubation (not shown).

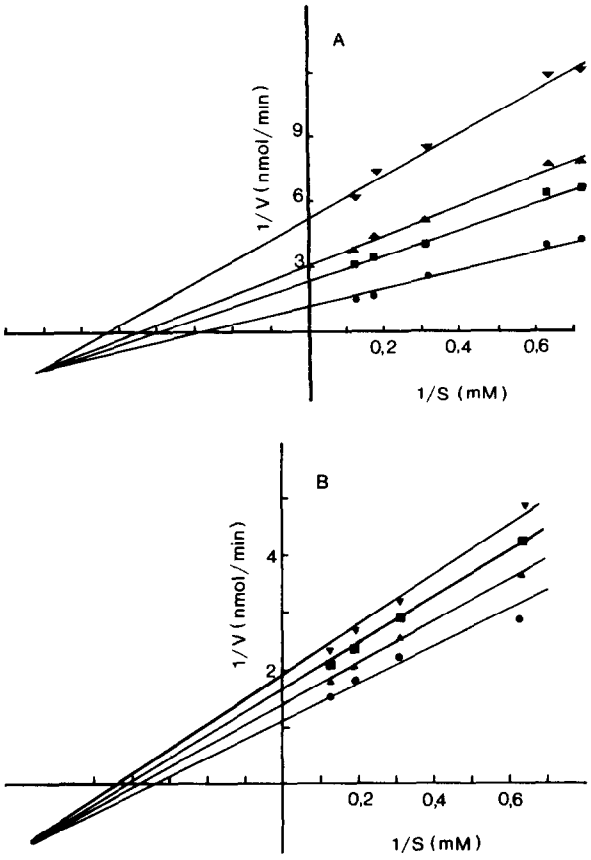

Fig.1. Lineweaver-Burk plots of the β-(1,3) glucan-synthase activity at several concentrations of: (A) papulacandin B ((●) control, (■) 1.3 μg/ml, (▲) 2.6 μg/ml, (▼) 13.1 μg/ml); and B aculeacin A ((●) control, (▲) 26 μg/ml, (■) 66 μg/ml, (▼) 132 μg/ml).

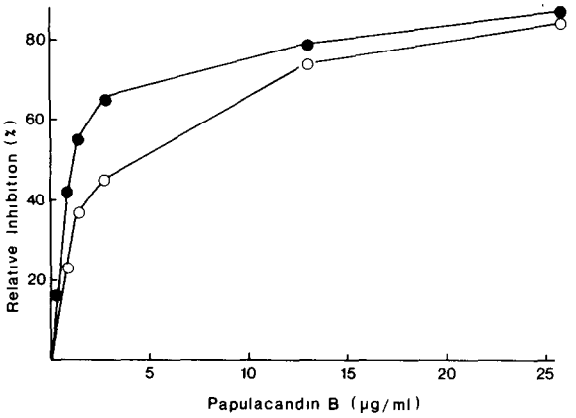

Fig.2. Inhibition of β-(1,3) glucan-synthase by papulacandin B in the presence (●) and absence (○) of GTP. Maximal activities with and without GTP are 14.3 and 4.7 nmol, respectively.

GTP, a typical activator of yeast glucan synthase [17], also stimulates the enzyme from *G. lactis*. The effect of papulacandin B, in terms of percentage inhibition, was the same either in the absence or in presence of GTP (fig.2). The same result was obtained by varying the order of addition of the antibiotic and GTP to the reaction mixture.

4. Discussion

The fact that the antibiotics inhibit the glucan-synthase 'in vivo' and that the inhibition is independent of the time of incubation suggests that aculeacin A and papulacandin B need not be metabolized or interact in a specific way with membrane enzymes in order to be active, as has been hypothesized [4]. On the other hand, the presence of the antibiotics in a growing culture renders the synthase (as measured 'in vitro') partially inactive. These results might be explained by assuming an irreversible attachment of the antibiotics to the synthase on the plasma membrane. A verification of this hypothesis will require the availability of radioactively labeled antibiotics.

The addition of papulacandin B increases the affinity of the enzyme for the substrate and on the other hand the activity cannot be driven to zero even in the presence of high amounts of antibiotic. This might be the behavior of an enzyme [16] in which the EI complex has higher affinity for the substrate than the enzyme alone, however the EIS complex, although

productive, would be processed at a lower rate than the ES complex. The situation for aculeacin A is more or less similar except that this antibiotic is a poorer inhibitor.

The antibiotics may interfere with intermediate steps of β-(1,3) glucan-synthase although no indication of the presence of lipid intermediates has been detected [8].

Papulacandin B does not counteract the stimulation of glucan-synthase by GTP, an indication that GTP and the antibiotic interact with the synthase at different sites. A more detailed study of the mechanism of inhibition must await a better knowledge of the system and its regulation [17] as well as the solubilization of the enzyme complex.

The β-glucan is the main structural component of *S. cerevisiae* cell wall whereas in ascomycetes fungi, i.e., *G. lactis*, not only β-glucan but chitin are the structural components [6]. When the synthesis of β-glucan is inhibited, the yeast cell would lyse, whereas in fungi having chitin the situation may be not so drastic since chitin could somehow compensate to a certain extent the defect in β-glucan synthesis. That might explain why the concentration of papulacandin B needed to lyse *S. cerevisiae* [2,4] is not sufficient to lyse *G. lactis*. Surprisingly however, the 'in vitro' inhibition of glucan-synthase from *S. cerevisiae* by papulacandin B is smaller than that described for *G. lactis* (A. D., unpublished). The significance of this result is unknown.

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