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P-FACTOR, A DEVELOPMENTAL HORMONE OF *PENICILLIUM CYCLOPIUM*?

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Key Word Index—*Penicillium cyclopium*; Aspergillaceae; Ascomycetes; biochemistry; benzodiazepine alkaloids; cyclopenin; sporulation; hormones; P-factor.

Abstract—From the mycelium of *Penicillium cyclopium* a biologically active fraction (P-factor) was isolated, which increases conidiation and the formation of the benzodiazepine alkaloids cyclopenin and cyclopenol. Its activity was determined by measuring the increase of alkaloid formation in strain SM 72. On a preparative scale P-factor preparations were obtained from fermenter-grown hyphae of mutant dev 63 by extraction with water at 120°. P-factor is strongly hydrophilic but it is not a protein. It was active if added during conidiospore germination and early growth phase, causing an acceleration of protein biosynthesis. The action on alkaloid biosynthesis and sporulation is indirect and resembles that of a developmental hormone.

INTRODUCTION

Low MW compounds involved in the co-ordination of cell differentiation and development (hormones) are well known in animals and higher plants. Compounds with similar biological activity have also been found in certain microbial cultures. A well-known example is the A-factor formed in *Actinomyces streptomycini* (a procaryote), which like animal or plant hormones, is dependent for its formation and activity on the developmental phases of the producer organism, is active in very small quantities, influences large areas of metabolism, i.e. the whole phase of cell specialization including streptomycin formation and sporulation, and shows no chemical relation to the processes it influences [1–7].

Penicillium cyclopium synthesizes alkaloids of the cyclopenin–viridicatin group and conidiospores during a phase of chemical and morphological cell specialization (idiophase) which follows the phase of hyphal growth (trophophase) (for summary see ref. [8]). The expression of alkaloid biosynthesis and conidiospore formation is triggered endogenously. The nature of the trigger(s) is unknown. However, a fraction was isolated from the mycelium of *P. cyclopium* which increases alkaloid formation and conidiation [9]. This paper reports on the properties and mode of action of this fraction, called P-factor, which shows several similarities to the A-factor of *A. streptomycini*.

RESULTS

Tests for activity

A biological method was developed to test P-factor activity. It is based on the increase of cyclopenin–cyclopenol biosynthesis in hyphae of *P. cyclopium* strain SM 72 after administration of P-factor. Surface cultures were grown by the batch method on a relatively simple nutrient solution (NL I) containing glucose, ammonium and phosphate as main constituents. Cyclopenin and cyclopenol released from the hyphae in the culture broth were assayed photometrically [10]. The alkaloid content was determined 7 days post inoculation (p.i.), i.e. at the time cyclopenium–cyclopenol concentration in the nutrient solution reached its maximum.

Fig. 1 shows some typical results of the biological test. There is a direct relationship between P-factor concentration in the nutrient solution and the increase of alkaloid formation at low P-factor concentrations. For calculations this straight and steeply rising part of the curve is used. The standard deviation within a series of 20 tests was ± 13%. It was smaller, if the alkaloid content was calculated per mg mycelial dry wt (± 7%), indicating that differences in the mass of hyphae cause much of the deviations. In experiments with different numbers of spores used for inoculation there was a linear correlation between alkaloid formation in the control cultures and the increase in alkaloid synthesis resulting from a given P-factor concentration. Activity is therefore expressed as percentage increase of alkaloid biosynthesis compared to control cultures. If calculated per mg dry wt the interassay reproducibility was ± 20% in 16 series assayed within 1 year.

Synthesis in cultures

P-Factor is a constituent of the cells of *P. cyclopium*. P-Factor activity was demonstrated in the wild-type strain SM 72 and in all developmental mutants tested, e.g. in the strain dev 63 [11]. No activity was found in the culture broth of the wild-type strain and the mutants or in cultures of several other microorganisms tested, e.g. *P. griseofulvum*, *P. notatum*, *Aspargillus niger*, *Saccharomyces cerevisiae* and *Actinomyces streptomycini*. Similar amounts of P-factor were extracted from young cells of surface and

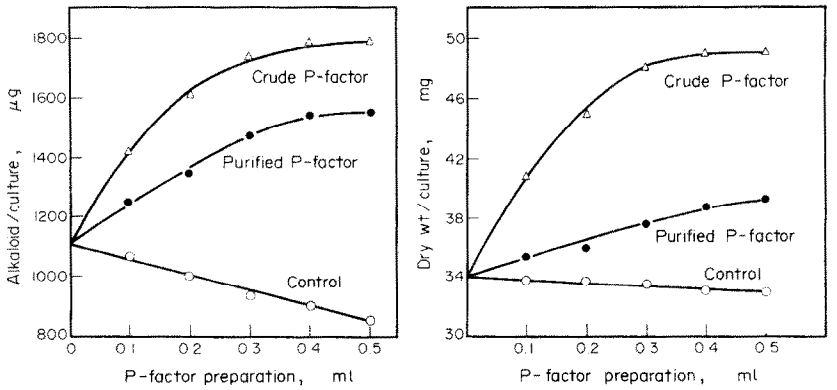

Fig. 1. Influence of P-factor concn on the formation of alkaloids and mycelial dry wt of *P. cyclopium* SM 72. The fungus was cultivated and assayed as given in Experimental. From the values obtained after addition of 0.1 ml of the P-factor-containing preparations the following P-factor activities were calculated: crude extract 124 units/ml, purified extract 128 units/ml. The decrease of mycelial dry wt and alkaloid concentration in the control cultures is due to the dilution of the nutrient solution with increasing amounts of water. The increase of dry wt after administration of purified P-factor is due in part to enhanced sporulation.

submerged cultures (Table 1). P-Factor was formed when cultures were grown in nutrient solutions NL I (main carbon source: glucose) and SM (main carbon source: sorbitol–mannitol), despite the fact that growth in NL I was much more vigorous than in SM, and that in contrast to the other variants [10, 12], submerged cultures grown in NL I synthesize neither alkaloids nor conidiospores.

In surface cultures on NL I the P-factor activity per mg cell material rapidly decreased with increasing age of the cultures. In submerged cultures this decrease was relatively small (cf. Table 2 for strain dev 63).

For the production of larger quantities of P-factor fermenter-grown cultures were used. In routine experiments mutant dev 63 [11] was cultivated in a 4 l. fermenter in NL I. Under these conditions strain dev 63 gave better yields per l. of culture than the wild-type strain due to a higher rate of dry mass formation and increased P-factor amount per mg dry wt. Addition of yeast extract to the nutrient solution increased growth of mutant dev 63 and consequently the amount of P-factor per l. of culture. To avoid contamination of the preparations with yeast constituents the pure nutrient solution NL I was used. Table 2 shows that the yield of P-factor after 4 days of cultivation approaches its maximum. Hence cultures were harvested at this time.

Extraction and purification

P-Factor can be extracted from cells of *P. cyclopium* with water after cell disintegration either by grinding with sand or heating. In routine experiments preparations were obtained by autoclaving cells at 120°. Addition of acetic acid, ammonia or sodium chloride caused no increase in yield. Sterile preparations were stable for long periods even at room temperature.

Crude P-factor preparations stimulated formation of alkaloids and conidiospores as well as dry wt of idiophase cultures (Fig. 1). However, most of the dry wt-stimulating substances were removed by ultrafiltration. With the use of membranes penetrated by compounds with a MW of up to *ca* 500 (Amicon Diaflo ultrafilter UM 05) P-factor remained in the supernatant, whereas a large part of the dry wt-promoting substances filtered through (Table 3). The filtrate contained most of the amino acids, sugars, phosphates, etc. present in the crude extract. Thus stimulation of dry wt by this fraction is an effect of better nutrition of the mould. The experiment also demonstrated that P-factor activity is not due to phenyl-

Table 1. P-Factor activity 66 hr p.i. in cells of *P. cyclopium* SM 72 grown under different conditions

Type of cultivation	Nutrient solution	Dry mass of cells (mg/ml culture)	P-Factor activity	
			units/mg dried cells	units/ml culture
Surface	NL I	8.8	0.80	7.0
	SM	1.3	0.70	0.9
Submerged	NL I	10.5	0.70	7.4
	SM	2.0	0.65	1.3

Table 2. P-Factor activity in fermenter-grown cultures of *P. cyclopium* dev 63 in NL I

Harvest of cells (hr after inoculation)	Dry mass (mg/ml)	P-Factor activity	
		units/mg dried cells	units/ml culture
55	5.6	0.96	5.4
72	9.8	0.90	8.8
96	13.6	0.85	11.6
144	16.0	0.80	12.8
240	10.0	0.65	6.5

Table 3. Purification of P-factor by ultrafiltration

Fraction	Dry wt (mg/ml)	P-Factor activity	
		units/ml	units/mg dry wt
Crude extract	43	122	2.9
Filter UM 10, supernatant	17	0	0
Filter UM 05, supernatant	7	115	16.4
Filter UM 05, filtrate	18	0	0

alanine, an amino acid which in higher concentrations may increase alkaloid biosynthesis under the experimental conditions of the biological test (cf. Discussion), but passes through filter UM 05. All P-factor activity was washed through Diaflo ultrafilter UM 10 which excludes substances of MW $> 10\,000$. It partly passed through Diaflo filter UM 2 which retains substances of MW $>$ *ca* 1000. These results indicated that P-factor is of medium MW and is not a protein. In routine experiments preparations were first washed through UM 10 filters and separated from low MW compounds by filtration through UM 05. By means of this procedure more than 80% of the substances accompanying P-factor activity in crude extracts were removed.

Biological activity

P-Factor preparations purified by ultrafiltration increased formation of cyclopenin–cyclopenol as well as synthesis of conidiospores in *P. cyclopium* SM 72. However, neither cyclopenase activity nor the content of melanin, parameters characterizing the maturity of the conidiospores[10, 13, 14], were influenced. In developmental mutants of *P. cyclopium* SM 72 with blocked alkaloid formation and/or sporulation, no normalization after addition of P-factor took place. With mutants still capable of synthesizing alkaloids and/or conidiospores, stimulation usually was of the same order of magnitude as that of the wild-type strain, but in some cases was absent.

The influence of P-factor on alkaloid formation and sporulation was similar in cultures of *P. cyclopium* grown on pure NL I and NL I supplemented with yeast extract and peptone. Substitution of glucose by galactose or saccharose and of ammonium by glycine, asparagine or glutamine showed no influence on activity. These results indicate that P-factor activity is relatively specific.

P-Factor preparations were active only when present at the beginning of the development of *P. cyclopium*, i.e. at germination of the conidiospores and the early growth phase of the hyphae. P-Factor added 48 hr p.i. showed only half of the biological activity and if added 96 hr p.i. or later it was without effect. There was only a short period of contact necessary between conidiospores and P-factor preparations for triggering the increase in alkaloid biosynthesis and sporulation. A 2 hr treatment of conidiospores with P-factor during the beginning of spore germination was sufficient to cause the biological effect.

The rate and speed of germination of conidiospores were enhanced after P-factor treatment. In addition it caused an acceleration of hyphal growth during the early stages of development of *P. cyclopium*. The dry mass of hyphae was *ca.* doubled after addition of P-factor 48 hr p.i. Even more distinct was the enhancement of protein content viz. the rate of protein biosynthesis (radioactivity in the TCA precipitate after feeding L-[U-^{14}C]phenylalanine and the accumulation of L-[^{14}U]phenylalanine in the TCA non-precipitable fraction. (For experimental details see ref. [10].) At the beginning of the period of alkaloid formation and conidiation, however, these parameters in the cultures treated with P-factor resembled those of the control cultures. In both types of cultures sporulation and alkaloid biosynthesis started at the same time, indicating that the acceleration of hyphal growth at the beginning of cultivation is not followed by a general acceleration of development.

DISCUSSION

P-Factor was found only in *P. cyclopium* and probably is a specific constituent of the cells. Its MW and biological action (taking place only during the initial development of the mould, i.e. at a phase prior to the beginning of cyclopenin–cyclopenol biosynthesis) indicated that P-factor is not a precursor of the alkaloids of *P. cyclopium*. Stimulation of alkaloid biosynthesis by P-factor in media supplemented with yeast extract or peptone, i.e. preparations containing various amino acids, vitamins, trace metals, etc. showed that the activity is not due to a special nutrient whose limitation is overcome by administration of P-factor. All experiments indicate that P-factor acts as a signal to the developmental programme of *P. cyclopium* and influences alkaloid formation and sporulation as a regulator (hormone). Several micro-organisms show a phase of competence at the beginning of the growth phase for signals influencing further development [15]. Alkaloid formation in *P. cyclopium* is stimulated at this phase by P-factor as well as by administration of the precursor amino acid phenylalanine and by certain phenylalanine derivatives which are not incorporated in cyclopenin–cyclopenol [9].

EXPERIMENTAL

Strains. P. cyclopium SM 72 [10], the wild-type strain, and dev 63, a mutant derived therefrom by NNMG treatment [11]. A large number of developmental mutants were prepared by UV-irradiation of conidiospores of strain SM 72 [11].

Nutrient solns. Cultures were grown at 24° in the dark in Petri dishes (diam. 14 cm) with 100 ml nutrient soln (surface cultures), in 500 ml flasks with 200 ml nutrient soln on rotary shakers (250 rpm) or in a fermenter (see below). Media were inoculated with conidiospores derived 5–6 days p.i. from surface cultures grown on nutrient soln NL I solidified with agar (*ca.* 10^6 spores/ml).

Determination of activity. Test tubes (diam. 18 mm) containing 3 ml NL I, 0.5 ml of a suspension of conidiospores of *P. cyclopium* SM 72 and 0.1, 0.2, 0.3, 0.4 and 0.5 ml, respectively, of the P-factor preparation to be tested or of H_2O (control) were prepared in triplicate and incubated at 24°. After 7 days the dry wt of the mycelium and the content of cyclopenin–cyclopenol in the medium were determined. P-Factor activity (given in units) was calc. from the formula

$$(a_p/m_p - a_c/m_c)100 = a_c/m_c,$$

where a = alkaloid content in medium of P-factor containing samples (a_p) or control (a_c) and m = mycelial dry wt of P-factor containing samples (m_p) or control (m_c).

Determination of biological parameters. Dry wt of mycelium, amount of conidiospores, cyclopenin–cyclopenol content of the culture medium, protein content, amino acid uptake and rate of protein biosynthesis were determined according to ref. [10].

Preparation of P-factor. P. cyclopium mutant dev 63 was grown for 4 days in NL I in a 4 l. fermenter (Ultraferm 1601, stirring at 300 rpm, aeration 1.2 l/min; temp. 24°). The mycelium was harvested by vacuum filtration, suspended in two parts of H_2O and the suspension heated to 120° in an autoclave. The mixture was filtered and the filtrate evaporated *in vacuo*. The residue was dissolved in 5 ml H_2O per g mycelial dry wt, the soln centrifuged and the clear supernatant (crude P-factor prepn) filtered through Diaflo ultrafilter UM 10 (Amicon) to 10% of its vol. The residue was dil. twice with nine parts of H_2O and again filtered. The filtrates were concd to the original vol. and filtered through Diaflo ultrafilter UM 05 (Amicon) to 10% of its vol. The residue was dil. with nine parts of H_2O and again filtered. The non-filtered part of the soln was dil. to the original vol. (purified P-factor preparation).

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