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Extracellular polypeptides of *Anabaena* L-31 : Evidence for their role in regulation of heterocyst formation

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Abstract. Extracellular polypeptides released by both N_2-grown [peptide I] and NO_3-grown [peptide II] *Anabaena* L-31 have molecular weight of approximately 3,500 but have distinctly different amino acid composition. Acid hydrolysis of the peptide I fraction (obtained by separation on Sephadex G-25) yielded ten amino acids whereas that from peptide II fraction yielded only 3 amino acids. On addition to a freshly inoculated N_2-grown culture, the peptide I fraction stimulated pro-heterocyst and to a lesser extent heterocyst differentiation, whereas the peptide II fraction strongly inhibited differentiation. The inhibitory effect of polypeptide II fraction could not be relieved by methionine sulphoximine, which by itself enhances differentiation, but was greatly relieved by addition of the peptide I fraction. The data suggest but does not prove, that *Anabaena* L-31 synthesises "inducer" or " inhibitor " peptides which could possibly control pattern formation.

Keywords. *Anabaena* L-31; blue-green algae; heterocyst differentiation; extracellular peptides; heterocyst regulation.

Introduction

Specialised cells called heterocysts are characteristic of filamentous blue-green algae which aerobically fix nitrogen. Using radioactive nitrogen gas [(^{13}N) N_2] it has been conclusively shown (Thomas *et al.,* 1977) that heterocysts are the sites of nitrogen fixation. Although initial observations of Fogg (1944, 1949), that heterocyst frequency is inversely related to cellular nitrogen content and that added nitrogenous compounds inhibit heterocyst formation, have been confirmed and extended in many laboratories (Fay *et al.,* 1954; Stewart, 1964; Mickelson *et al.,* 1967; Talpasayi and Kale, 1967; Ogawa and Carr, 1969; Thomas and David, 1971; David and Thomas, 1972; Kulasooriya *et al.,* 1972; Bradley and Carr, 1976) the physiology of heterocyst regulation is still far from clear. Nitrate (Thomas and David, 1971) or ammonium ions (Stewart and Rowell, 1975) *per se* do not inhibit heterocyst formation but a product of ammonia assimilation is the putative (Stewart and Rowell, 1975; Thomas *et al.,* 1977) inhibitor.

It has been proposed (Fogg, 1949) that heterocysts produce a substance which diffuses along the filaments and inhibits the formation of other heterocysts. Evidence in support of this hypothesis (Wolk, 1967) and an extension of it (Wilcox

et al., 1973a, b) has been obtained. The identity of the inhibitory substance is unknown but 7-azatryptophan (Mitchison and Wilcox, 1973) and rifampicin (Wolk, 1975) have been shown to relieve the inhibitory effect.

Blue-green algae excrete nitrogenous products, principally peptides (Fogg, 1952; Whitton, 1965; David and Thomas, 1972; Walsby, 1974). Although a great deal of information indicating the involvement of extracellular peptides in bacterial differentiation is available (see Payne, 1976; Katz and Domain, 1977), the role of such peptides in blue-green algal differentiation has not been critically examined. In an earlier communication we showed (David and Thomas, 1972) that the peptide(s) released by *Anabaena* L-31 under N_2-fixing condition (favouring heterocyst production) have distinctly different amino acid composition from that released by the alga growing in the presence of nitrate (unfavourable for heterocyst production). In this paper we report further characterisation of the extracellular peptides and their differential effect on the production of heterocysts in *Anabaena* L-31.

Methods

Culture conditions

Anabaena L–31, a non-sporulating paddy field blue-green alga (Thomas and David, 1972) available in axenic condition was the experimental organism. The alga was grown at 29 ± 1°C in 11 Ehrlenmeyer flasks containing 500 ml Cyanophycean medium of the following composition (g/1) : $MgSO_4 \cdot 7H_2O$, 0·25; K_2SO_4, 0·625; $Na_2HPO_4 \cdot 2H_2O$, 0·50; Na_2EDTA, 0·25; $CaCl_2 \cdot 2H_2O$, 0·042; H_3BO_3, 0·057; $FeSO_4$ $7H_2O$, 0·025; $ZnSO_4 \cdot 7H_2O$, 0·044; $MnCl_2 \cdot 4H_2O$, 0·02; MoO_3, 0·004; $CuSO_4 \cdot 5H_2O$, 0·008; $CoSO_4 \cdot 7H_2O$, 0·003. KNO_3 (0·625) replaced K_2SO_4 when the alga was to be grown in the presence of nitrogenous compound. The flasks were provided with aeration at the rate of 2 1/min and 5000 lux white light from a bank of cool fluorescent lamps.

Separation of extracellular peptides

Seven day-old algal suspensions (which did not reveal any cell lysis on microscopic examination) were sedimented and the supernatant solution was filtered to remove all algal filaments. The culture filtrate was lyophilised and dispersed in sterile water. Insoluble salt residue was removed by centrifugation. Portions of the supernatant solutions were loaded on Sephadex G-25 columns (50cm × 2·5 cm) and eluted with water. Fractions (1·5 ml) were collected using an automatic fraction collector. Absorbance (280 nm) of the fractions was measured using a Perkin Elmer 124 spectrophotometer. Actinomycin D, bacitracin, glucagon and insulin (all obtained from Sigma Chemical Co., London, UK) having M_r of 1247, 1411, 3647 and 5733, respectively were used as standards to determine the elution pattern on Sephadex G-25 in relation to the peptides. Fractions of polypeptides forming discrete peaks were pooled and stored frozen. Aliquots of the pooled fractions were analysed for total Kjeldahl nitrogen (Steyermark, 1961) and also hydrolysed with 6N HCl and amino acid content was determined using an automatic Beckman Unichrom amino acid analyser.

Assay for the effect of peptides on heterocyst production

Polypeptide fractions obtained on Sephadex gel filtration of either N_2-grown [peptide I] or NO_3-grown [peptide II] culture filtrate were added to freshly inoculated, N_2-grown, aerated test-tube cultures of *Anabaena* L–31 to yield a final concentration of 0·01 mg N polypeptide/ml of suspension. Pro-heterocyst and heterocyst frequencies, and nitrogenase activity were determined at 24 h intervals as described earlier (Thomas and David, 1972). Growth was assessed by determineing chlorophyll content (Mackinney, 1941). In some experiments peptides II and I were added in the ratio of 1 :1 and 1:1·5 respectively. One μM L-methionine-DL-sulphoximine (Sigma Chemical Co., London, UK), was present in certain assay experiments using peptide II.

Results

Only a single peak of material absorbing at 280 nm was obtained when the concentrated culture filtrate from either NO_3-grown or N_2-grown *Anabaena* was eluted from a Sephadex G-25 column (figure 1). The peaks in both the cases were symmetrical and eluted just after glucagon (M_r 3647) which was one of the four markers. The peak fractions obtained from both the filtrates were pink in colour and had an

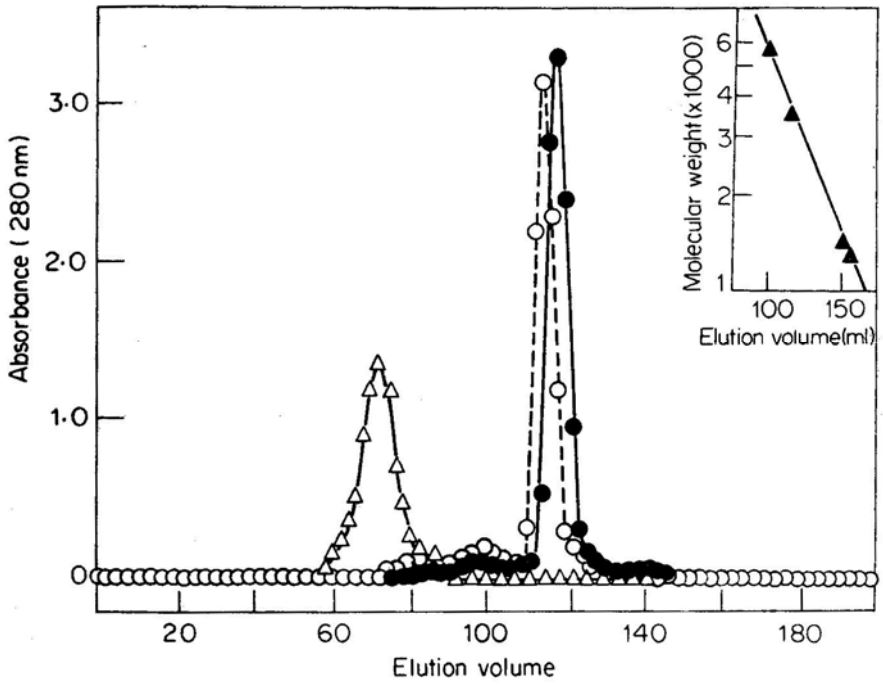

Figure 1. Elution profiles on Sephadex G-25 column of the extracellular polypeptides of N_2-grown (O) and NO_3-grown (●) cultures on *Anabaena* L–31. Blue dextran (Δ) was used to measure void volume. Inset : The peak elution volumes for insulin, glucagon, bacitracin and actinomycin D(M_r 5733, 3647, 1411 and 1247 daltons respectively) are plotted to yield a standard curve with respect to M_r.

absorbance maximum at 530 m The linear relationship (figure 1 inset) of elution volume to logarithm of *Mr* of the standards used indicate the efficiency of the Sephadex G–25 column.

The peptide fraction separated from the culture filtrates of N_2–grown *Anabaena* [peptide I] yielded 10 amino acids (table 1). The peptide fraction similarly obtained

Table 1. Amino acid composition of extracellular polypeptide fractions of *Anabaena* L–31 resolved on *Sephadex* G–25 column.

Amino acids	μmol/75 ml culture filtrate	
	N_2-grown	NO_3-grown
Aspartic acid	0·38	—
Threonine	0·19	—
Serine	0·21	—
Glutamic acid	0·27	—
Proline	—	—
Glycine	0·62	2·4
Alanine	0·22	—
Valine	0·15	—
Methionine	—	—
Isoleucine	0·12	—
Leucine	0·16	—
Tyrosine	—	—
Phenylalanine	0·11	—
Lysine	—	4·8
Histidine	—	—
Arginine	—	—
'X' amino acid*	0·00	16·2

– Denotes amino acids not detected or present in trace quantities, *i.e.*, less than 0·05 μmol.

* This amino acid has not been yet identified. The amino acid elutes at 22 min (just prior to cysteic acid) from the long column of the analyser.

from culture filtrates of the NO_3-grown alga [peptide II] contained only glycine, lysine and an unidentified acidic amino aicd which eluted just before cysteic acid on the long column of the amino acid analyser (table 1).

A distinct increase in differentiation of proheterocysts was observed in the presence of peptide I. Although, the increase in heterocyst differentiation was somewhat less distinct, the values obtained were consistently higher in the presence of peptide I than in controls (figure 2). Addition of peptide II severely inhibited differentiation (figure 2). Concordantly, there was an increase and decrease in nitrogenase activity in the presence of peptide I and peptide II respectively (table 2). The % increase in nitrogenase activity was however much more than that of heterocysts. Although the presence of the glutamine analogue, L-methionine-

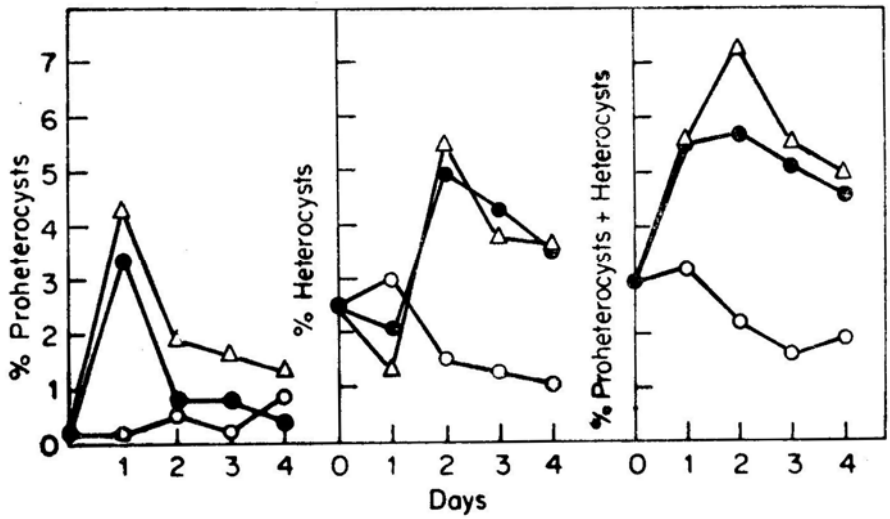

Figure 2. Effect of the addition of extracellular polypeptide fractions obtained from N_2-grown (Δ) and NO_3-grown (O) *Anabaena* L-31 on differentiation of proheterocysts and heterocysts in a freshly inoculated N_2-grown culture of the alga compared to control (●).

Table 2. Effect of extracellular polypeptides on growth, heterocyst differentiation and nitrogenase activity of *Anabaena* L-31.

Treatment	Growth (μg chl *a* ml^{-1})	Heterocyst (%)	Nitrogenase activity [μmol h^{-1} (mg chl *a*)$^{-1}$]
Control	2·30 (100)	5·0 (100)	2·09 (100)*
Polypeptide I	1·35 (57)	5·5 (110)	3·67 (175)
Polypeptide II	3·90 (170)	1·2 (24)	0·69 (33)

Chl, Chlorophyll
The observations were made 72 h after addition of the peptides to freshly inoculated N_2-grown cultures.

* Values in paranthesis denote per cent of control values.

DL-sulphoximine, enhanced heterocyst differentiation (figure 3), addition of peptide II inhibited heterocyst formation as severely as in the cultures without methionine sulphoximine. However, the inhibitory effect of peptide II on proheterocyst formation could be almost fully relieved and that on heterocysts partially relieved by the addition of peptide I (figure 4), the reversal of inhibition being more at higher concentration of peptide I. These effects of the peptides were found to be (statistically significant (table 3). When the polypeptide II fraction was added

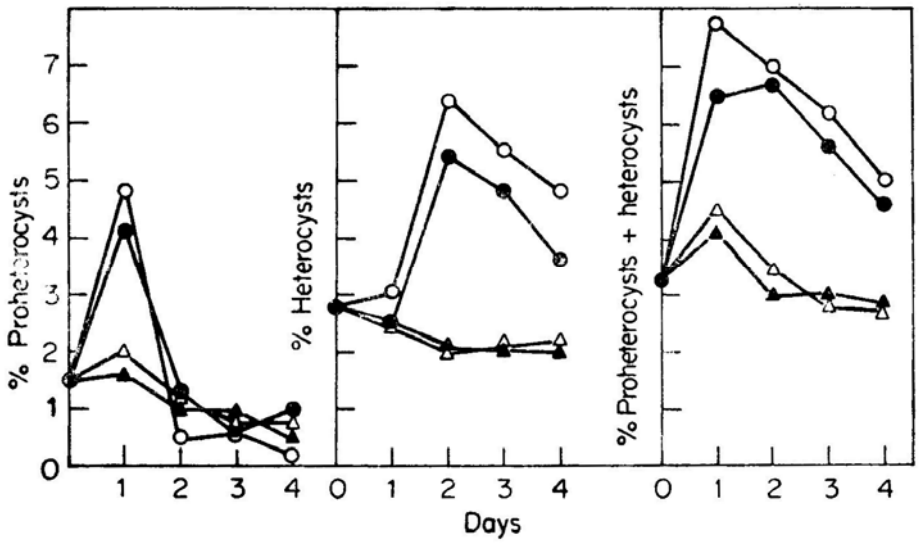

Figure 3. Effect of the addition of extracellular polypeptide fraction obtained from NO_3-grown *Anabaena* L-31 and L-methionine-DL-sulphoximine on differentiation of proheterocysts and heterocysts in a freshly inoculated N_2-grown culture of the alga. (●) control; (O) with L-methionine-DL-sulphoximine (▲) with polypeptide fraction; (Δ) with polypeptide fraction and L-methionine-DL-sulphoximine.

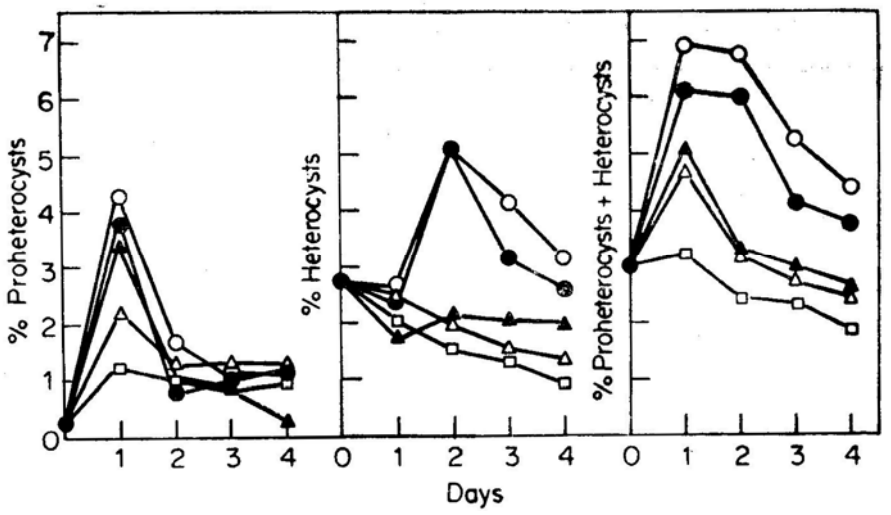

Figure 4. Effect of addition of extracellular polypeptide fractions obtained from NO_2-grown [peptide I] and NO_3-grown [peptide II] *Anabaena* L-31 on differentiation of proheterocysts and heterocysts in a freshly inoculated N_2-grown culture of *Anabaena* L–31. (●) control; (O) peptide I; (□) Peptide II; (Δ) 1 part peptide II and 1 part peptide I; (▲) with 1 part peptide II and 1·5 parts peptide I.

to a freshly inoculated N_2-grown culture of *Anabaena* growth was enhanced by about 70% (table 2) but addition of the peptide I fraction inhibited growth by nearly 40%.

Table 3. Effect of extracellular polypeptide fractions on differentiation of heterocysts.

Treatment	Total cell number	Proheterocyst + Heterocyst percent
Control	1673	6·20
0·01 mg/ml peptide II	2166	3·09
0·01 mg/ml peptide I	1727	6·93
0·01 mg/ml peptide II and 0·01 mg/ml peptide I	1459	4·66
0·01 mg/ml peptide II and 0·015 mg/ml peptide I	1323	5·92
C.D. ($p = 0·05$)		0·3
C.D. ($p = 0·01$)		0·5

Discussion

Although the extracellular peptides released in NO_3-grown and N_2-grown cultures of *Anabaena* L–31 have nearly the same M_r, their amino acid composition is distinctly different (table 1). We did not observe large extracellular pigment peptides M_r > 5000) similar to those found by Walsby (1974) in *Anabaena cylindrica.* The relatively young cultures (7 day-old) used in our experiments are not strictly comparable to the old cultures (4–6 weeks) which Walsby examined. Peptides are known to bind metals (Walsby, 1974) and the pink colour of the peptides may have been due to binding with cobalt in the medium. A solution of cobalt sulphate gave an absorption spectrum in the visible region similar to that of the peptide.

Experimental evidence from the laboratory of Wolk (1967; 1975; Wolk and Quine, 1975) supports the simple threshold model (Fogg, 1949) for heterocyst differentiation which postulates that concentration gradients of an inhibiting substance produced in heterocysts arise along the length of the filament and heterocysts are formed at the points of lowest concentration. Wilcox *et al.* (1973a, b) have proposed a combination of the inhibitory zone mechanism and an interactive mechanism, the latter postulated as the "support" needed by a proheterocyst from adjacent vegetative cells for its full development. The identity of these competing inhibitory and supporting compounds is not known. Because heterocysts can be totally eliminated from *Anabaena* in the presence of nitrate (Thomas and David, 1971) it follows that the inhibitor is present in abundance in the nitrate-grown cultures. Conversely, in young N_2-grown cultures, the substance promoting heterocyst formation would be present in adequate concentration. The peptide II and peptide I fractions which differentially affect heterocyst formation in *Anabaena* L–31 are apparently such compounds. Addition of the peptide II fraction clearly inhibited proheterocyst and heterocyst differentiation, whereas in the presence of peptide I fraction more differentiation occurred (figure 2). The statistically significant reversal of the inhibitory effect of peptide II by peptide I (table 3) together

with the data on the enhancement in the ability of peptide I to relieve the inhibition caused by peptide II at higher concentration (figure 4) indicate the competitive role of these peptides in heterocyst differentiation. The fact that the pronounced relief from the inhibitory effect on proheterocyst formation is not fully manifested in heterocyst production suggests sequential phases in the development and control of differentiation observed by Bradley and Carr (1977). Apparently, in the continued presence of peptide II, a significant number of the proheterocysts revert to the vegetative phase.

Nitrogenous compounds inhibit heterocyst formation. It is, therefore, significant that peptide II inhibits, whereas the peptide I enhances heterocyst formation. The inhibitory effect of peptide II is observed even in the presence of methionine sulphoximine indicating that reassimilation of ammonium possibly released by hydrolysis of the peptide is not involved in such inhibition. However, the observation that addition of peptide I enhanced growth (table 2) suggests that it may be metabolised after being broken down to its constituent amino acids. It is, therefore, possible that the inhibition is mediated through one or more of the amino acids and is not directly effected by the peptide.

Extracellular peptides assumed to be involved in bacterial differentiation (Payne, 1976; Katz and Demain, 1977) exhibit antibiotic and self-inhibitory properties. We have observed that the peptide fraction which stimulates heterocyst differentiation in *Anabaena* decreases its growth by nearly 40%. Jakob (1954, 1957) found that extracellular products of *Nostoc muscorum* cultures with large number of heterocysts had antibiotic activity whereas cultures without heterocysts did not. It was shown that 7-azatryptophan (Mitchison and Wilcox, 1973; Agarwal and Kumar, 1978) and rifampicin (Wolk, 1975) increase heterocyst frequency in *Anabaena.* Rifampicin is known to inhibit DNA-dependent RNA polymerase and it is tempting to speculate that the peptide I fraction from *Anabaena* L-31 which mimicks the rifampicin effect probably has a similar mechanism of action.

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