Profect 349

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R&D Project 0349
Field Validation of Algal Toxin Test Kit

Progress Report for Period
November 1991 - January 1992

University of Dundee
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CONTENTS

SUMMARY

1. Microcystin-LR purification has been established and the purified toxin has been supplied to Biocode to date.

2. Toxin has been supplied at two levels of purity:

   a 91-93%. This was understtod to be of sufficient purity for the purposes for which it was required.
   b 97-98%. This is the routine level of purity attained.

3. According to the Biocode estimate of 5 November 1991 of the amounts of toxin required and of the timing of requirements, toxin provision has been prompt and sufficient.

4. Unscheduled requests for toxin have been received although it has been possible to supply material between 1 and 6 days after receiving requests.

5. A laboratory procedure has been developed for the release of microcystin-LR from intact cells of *Microcystis aeruginosa* using small quantities of toluene. Optimization studies are in progress.

1. OBJECTIVES

1.1 Overall project objectives

To validate for the NRA the development and performance of the field test kit for microcystin-LR (MC-LR) developed by Biocode and develop field procedures for its use by NRA staff.

1.2 Specific objectives

The specific objectives to commence in the present reporting period were as follows:

- Liaise with and provide technical advice to Biocode Ltd.
- Purify microcystin-LR (MC-LR) and supply Biocode Ltd for the production and screening of monoclonal antibodies (MAbs).
- Inspect and assess work undertaken by Biocode Ltd.
- Develop methods for cyanobacterial cell lysis in water.

2. ESTABLISHMENT OF PROJECT AND APPOINTMENT

The project commenced with the appointment of Dr Steven G. Bell as a full-time Post-doctoral Research Assistant on 1 November 1991.

To enable Biocode Ltd to be supplied with purified MC-LR without delay, University staff were temporarily assigned to the cultivation of toxic *Microcystis* cells and purification of the toxin for some weeks before the contract was signed. This enabled 10 mg of MC-LR to be supplied to Biocode on 22 October 1991 at the start of the Project.

3. RESEARCH PROGRAMME

3.1 Technical progress

Work has progressed as per the schedule agreed with the NRA in the Memorandum of Agreement for Research Contract NRD 040.

Since the initial 10 mg of purified MC-LR were dispatched, a further 21.6 mg of the toxin have been purified and sent to Biocode Ltd following individual requests

(Table 3.1.1). The structure of MC-LR and a typical HPLC chromatogram of the purified toxin before dispatch are presented in Figures 3.1.1 and 3.1.2.

The purity of the toxin batches can be grouped into 2 categories:

a. 91-93%. Early batches were supplied at this level since it was indicated by Biocode that this level of purity would be sufficient for the procedures to be performed.

b. 97-98%. Microcystin-LR has otherwise been supplied at 97% and 98% purity. This level of purity can be achieved routinely.

Cyanobacterial cultures have been established for the production of other microcystin variants for later testing.

3.2 Lysis of cyanobacterial cells

In order to use microcystin antibodies for the optimal detection (and quantification) of the toxin(s) in water samples, it is necessary for the toxin to be released from cyanobacterial cells, if it remains in an intracellular form in the samples, to be tested. Procedures for the lysis of cyanobacterial cells for the release of toxin are being investigated.

Three treatments were initially examined using cultures of *M. aeruginosa* strain 7813. As an indicator of cell leakage, the appearance of the high molecular weight, water-soluble blue pigment phycocyanin in the water after cell removal has been initially monitored. Pigment release is measured as absorbance at 620 nm. As shown in Table 3.2.1, only toluene treatment was found to be potentially useful. In this case, toluene addition to a final concentration of 10% (v/v) to the *Microcystis* cell suspension in water, with mixing, resulted in the appearance of phycocyanin in the water.

The leakage of *M. aeruginosa* cells was subsequently investigated at a range of lower concentrations of toluene. As shown in Table 3.2.2, phycocyanin release was maximally achieved at a final concentration of 0.2 - 0.3% (v/v) toluene.

When 0.3% (v/v) toluene-treated cells were examined under the microscope about 75% of the cells were disrupted after 5 min, 95% after 10 min and the 100% after 15 min incubation. However, after only 5 min incubation, the cell-free phycocyanin concentration was as its maximum (Table 3.2.3), indicating that permeabilization and pigment (protein) release occurred before all of the cells appeared to be disrupted.

A cell-free supernatant of *M. aeruginosa* 7813 incubated with 0.3% toluene for 15 min was analysed by HPLC for MC-LR content. This was compared with an acetic

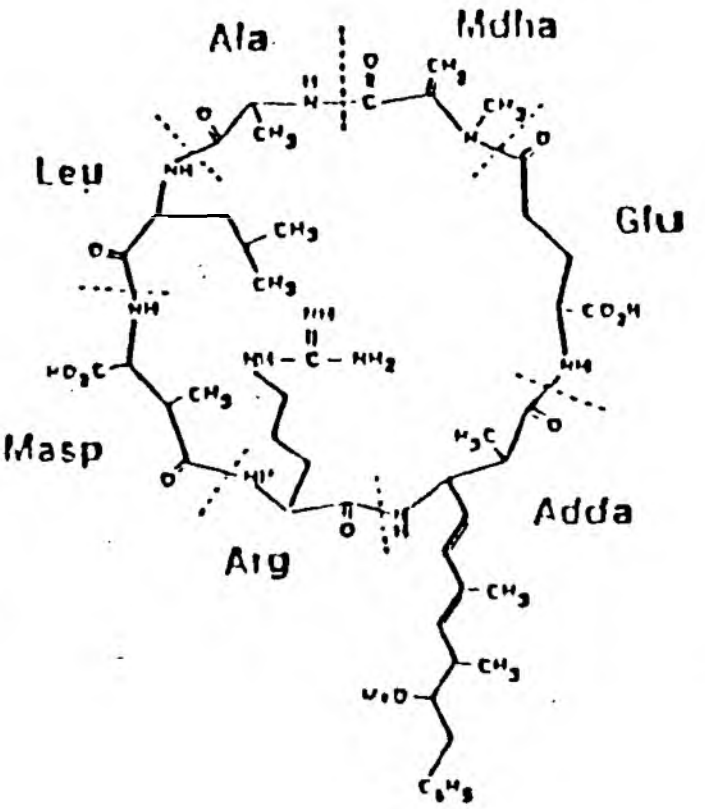

Figure 3.1.1 Structure of microcystin-LR

Abbreviations:

Masp	ß-methylaspartic acid
Leu	leucine
Ala	alanine
Mdha	N-methyldehydroalanine
Glu	glutamic acid
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
Arg	arginine

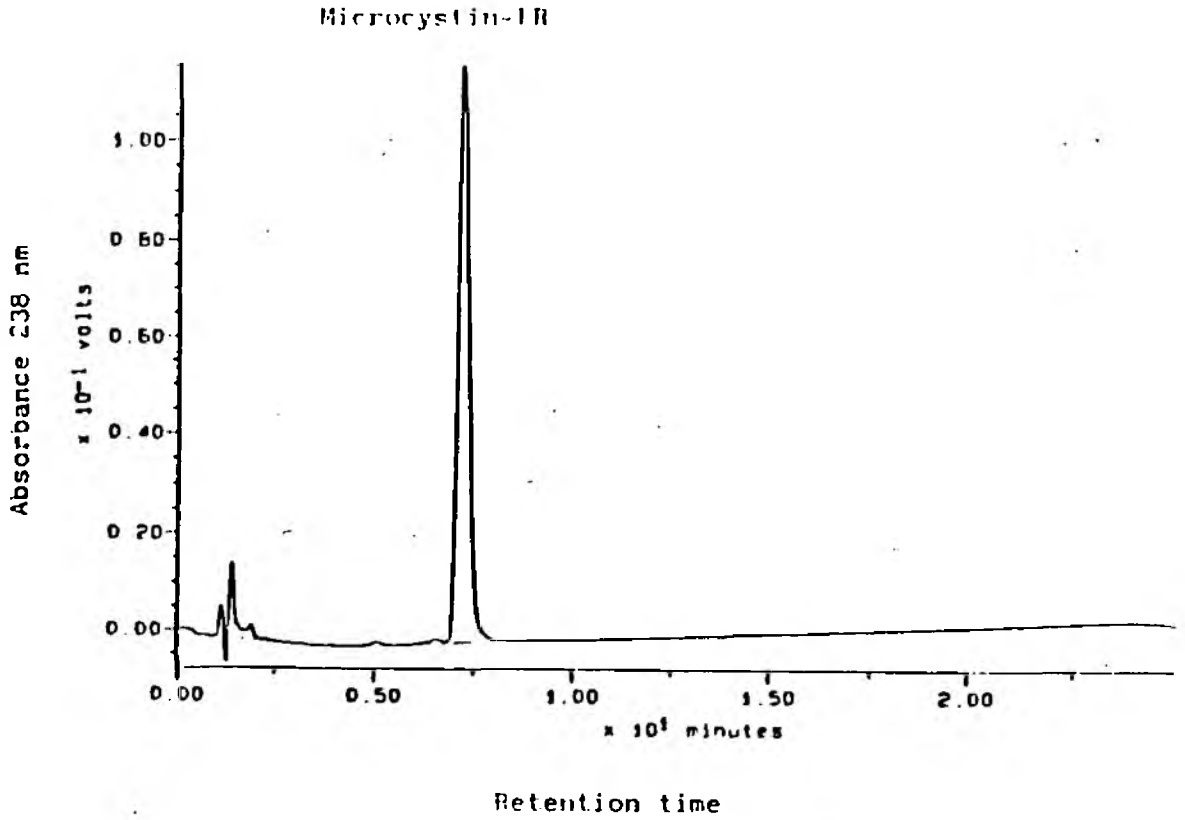

Figure 3.1.2 Typical HPLC chromatogram of purified microcystin-LR supplied to Biocode Ltd as in Table 3.1.1.

Table 3.1.1 Consignments of purified microcystin-LR dispatched to Biocode Ltd

MC-LR Lot No.	Amount Dispatched	Date Dispatched
Initial supply	10.0 mg	22.10.91
SGB 251191	5.0 mg	26.11.91
SGB 251191.2	4.5 mg	02.12.91
SGB 281191	4.5 mg	02.12.91
SGB 221191	3.6 mg	04.12.91
SGB 021291	4.0 mg	08.01.92
Total	31.6 mg	

Table 3.2.1 The release of phycocyanin (absorbance at 620 nm) from *Microcystis aeruginosa* cells by various agents

Addition	Final concentration (v/v)	Incubation time	Abs 620 nm cell-free supernatant[1]
None	-	-	0.000
Acetic acid	10%	48 hr	0.000
Sodium-hydroxide	10%	48 hr	0.000
Toluene	10%	5 min	0.144

1, cell suspension in water incubated with the reagent for the time stated then centrifuged at 14,000 rpm for 5 minutes in a microcentrifuge.

Table 3.2.2 The release of phycocyanin from *Microcystis aeruginosa* cells in water using toluene

Final concentration of toluene (v/v)[1]	Concentration of cell-free phycocyanin (μg per ml)[2]
0.0%	0.00
0.1%	0.00
0.2%	23.15 ± 0.23
0.3%	23.28 ± 0.22
0.4%	23.22 ± 0.38
0.5%	21.97 ± 1.77

1, toluene was added to the final concentration stated and the mixture shaken intermittently for 20 min.

2, after toluene-treatment, the cell suspension was centrifuged for 5 min at 14,000 rpm in a microcentrifuge and absorbances measured in the cell-free supernatant at 615 nm and 652 nm. The phycocyanin concentration was calculated as follows:

conc. of phycocyanin (μg per ml)

$$= \frac{Abs_{615} - (0.474 \times Abs_{652})}{0.00534}$$

Table 3.2.3 Time-course of the release of phycocyanin from *Microcystis aeruginosa* 7813 during treatment with toluene (0.3% v/v)

Incubation time (min)	Concentration of cell-free phycocyanin (μg per ml)[1]
0	0.197 (± 0.000)
5	17.331 (± 0.491)
10	16.731 (± 0.881)
15	13.198 (± 0.652)

1, see legend to Table 3.2.2

Table 3.2.4 Comparison of the release of MC-LR from *Microcystis aeruginosa* 7813 into water by toluene treatment, versus MC-LR from an equivalent amount of freeze-dried cells using acetic acid extraction.

Source and extraction procedure	Total yield of MC-LR[1]
Aqueous cell suspension; 0.3% toluene	53.49 µg
Freeze-dried cells; 5.0% acetic acid	49.63 µg

1, analysis was carried out using a C_{18} reversed phase HPLC column and water/acetonitrile/trifluoroacetic acid solvents.

acid extract of an equal amount of freeze-dried cells from the same culture (part of our procedure for the extraction and purification of MC-LR from dry cells). The results (Table 3.2.4) indicated that at least an equivalent amount of MC-LR was released from toluene-treated cells into water, compared to the amount of toxin extracted from an equivalent quantity of acid-treated freeze-dried cells.

It has been subsequently shown that *M. aeruginosa* cells trapped by passage of an aqueous cell suspension through a glass fibre-filter (Whatman GF/C), then incubated with 0.3% (v/v) toluene, release a similar amount of MC-LR as freeze-dried, acid-treated cells. The filter plus entrapped cells were placed in the toluene/water suspension and mixed intermittently for 15 min. Within this incubation period, the filter disintegrated, thus easing the release of the permeabilized cells into suspension. The resulting slurry of glass fibre and treated cell suspension was passed through a syringe plugged with glass wool, the filter material and majority of cell particulates being retained within the syringe. The cell-free solution passed through the syringe and was analysed by HPLC for MC-LR content.

3.3 Future programme

The specific objectives for the reporting period February 1992 - April 1992 are to continue the work objectives begun during the present reporting period (see section 1.2).

4. DISCUSSION

The work has begun and is progressing well. The supply of MC-LR to Biocode has progressed without delay even though the quantities required (total to date, 31.6 mg) have been considerably greater than was originally budgeted for when the contract was drawn up. These large quantities of purified toxin have required large-scale culture of *M. aeruginosa* as well as substantially more time on toxin purification than initially envisaged. A schedule of quantities and dates of microcystin-LR requirements was received from Dr D. Baron on 5 November 1991. According to these estimates, toxin supply has been both prompt and sufficient. Since the amounts of toxin required have been higher than anticipated this may lead to spending over budget.

It has been possible to meet unscheduled requests for toxin within 1 to 6 days of receiving a telephone request. Although unscheduled requests for toxin have not affected progress on other aspects of the work, the latter may be adversely affected if further unscheduled increases in the need for toxin occur. Culture of cyanobacteria which produce MC-LR-related toxins has begun.

Studies on the lysis of cyanobacterial cells to increase the availability of microcystin in various water samples for binding by antibodies in the prospective test are progressing well. A method involving the use of toluene to permeabilize cells is under investigation. Filtration of the cells from water samples by glass fibre filters and the subsequent permeabilization of cells with toluene has been achieved. This may have favourable implications for the separate measurement of microcystin(s) in the water and in the cyanobacterial cells.

It is understood from Biocode that toluene at the low levels used (e.g. 0.3% v/v) should not adversely affect the binding of toxin to antibodies in the columns to be developed. However, if this were the case, a remedy would be possible: the filter supporting the cells may be placed in a small volume of 0.3% toluene which would then be diluted after the cells had been permeabilized, thus reducing the toluene concentration. Work in future will include investigations of filter loading with cyanobacterial cells, optimum volume of toluene suspension and the use of cyanobacteria from diverse groups which can produce microcystins. Further lysis procedures will also be investigated.

5. FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

Toxin purification according to a schedule of times and quantities is necessary to allow the other essential work on cyanobacterial cell lysis for toxin release, comparative studies and the production of additional toxin variants to proceed on time. If unforeseen increases occur in the amounts of toxin needed, then these may delay the completion of the development of the lysis and assay procedures.

6. COST OF WORK CARRIED OUT IN THE PERIOD NOVEMBER 1991 - JANUARY 1992

The cost of the work carried out in the first three months of the project (1 November 1991 - 31 January 1992) is summarised below alongside the 1991/1992 and total project budgets:

	This period (£)	1991/1992 Total (£)	Project Total (£)
Staff salaries and overheads	8,982	27,500	57,938
Travel and subsistence	99	1,000	1,800
Laboratory consumables	250	4,000	6,000
Total	£9,331	£32,700	£66,738

7. ESTIMATE OF COST OF WORK FOR THE PERIOD FEBRUARY 1992 - APRIL 1992

It is estimated that the work to be carried out between 1 February 1992 and 30 April 1992 will cost the following:

	£
Staff salaries and overheads	10,000
Travel and subsistence	300
Laboratory consumables	1,000
Reports	166
Total	£11,466