Microalgae and Cyanobacteria as a Source of Glycosidase Inhibitors

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Culture filtrates and organic solvent extracts of over 500 freshwater and marine eukaryotic microalgae and cyanobacteria were screened for the presence of glycosidase inhibitors. Rapid colorimetric assays were used to detect inhibitors of α -glucosidase, α -amylase and β -galactosidase. Inhibitors were found from 38 species. The results suggest that microalgae and cyanobacteria have potential as a source of glycosidase inhibitors which may have clinical applications.

INTRODUCTION

Many disease states are mediated by excessive enzyme activity, and inhibition of the enzymes involved could lead to treatment and control of the disease. Enzyme inhibitors have potential value therefore, as clinical tools. In the early 1960s, Umezawa began screening actinomycetes for the presence of enzyme inhibitors with a view to possible clinical or research use, and since then many dozens have been described (Umezawa, 1972, 1982). This screening has continued, but has concentrated mainly on the actinomycetes. It was decided to extend the search for potentially clinically useful enzyme inhibitors to a group of micro-organisms which in this respect are relatively unstudied – the microalgae. Like bacteria and fungi, microalgae are amenable to study under controlled batch culture conditions, where the effects of variation of chemical and physical parameters may be determined, and the production of any interesting compound optimized.

It has long been recognized that inhibition of glycosidases could be of value in the treatment and prevention of conditions such as diabetes, obesity, hyperlipoproteinaemia and hyperlipidaemia. These could all be better managed by controlling the kinetics of intestinal carbohydrate digestion and monosaccharide absorption, thus regulating blood sugar levels, insulin response, etc. The search for glycosidase inhibitors from natural sources has been going on for some time and many interesting and potentially valuable structures have been uncovered, e.g. acarbose and nojirimycin (Puls *et al.*, 1977; Muller, 1985). Algae, particularly marine macroalgae, have been screened for antibiotic activity (Burkholder *et al.*, 1960; Hornsey & Hide, 1974; Pesando & Caram, 1984; Reichelt & Borowitzka, 1984), but not for enzyme inhibitors. We therefore decided to screen a range of eukaryotic microalgal and cyanobacterial culture filtrates and organic solvent extracts for the presence of inhibitors to α -glucosidase, which hydrolyses 1,4- α -glucosidic linkages, and rather more specifically, to α -amylase, an endo-1,4- α -glucanase acting on polymers, and also to β -galactosidase, which hydrolyses 1,4- β -glucosidic linkages.

METHODS

Organisms. The algae and cyanobacteria were obtained mainly from the Culture Centre of Algae and Protozoa, Cambridge, UK. Approximately 300 freshwater and 200 marine eukaryotic algae, and 70 freshwater and 10 marine cyanobacteria, were cultured. All cultures were unialgal, but were not tested for axenicity. However, any obviously contaminated cultures were discarded.

The freshwater eukaryotic algae were all grown in modified Chu 10, a minimal medium designed for autotrophic growth (Asher & Spalding, 1982); many were also grown in M1, a nutrient-rich medium based on proteose peptone (Asher & Spalding, 1982). The marine eukaryotic algae were all grown in medium M11, (Asher & Spalding, 1982), and the cyanobacteria were all grown in medium BG-11 (Vonshak, 1986).

Cultures were inoculated from slopes into 250 ml conical flasks containing 100 ml medium, incubated at 23 °C, shaken at 100 r.p.m. with a 16/8 h (light/dark) photoperiod and a light intensity of 3000 lx. The cultures were harvested after 6 weeks by centrifugation. The culture supernatant was decanted and the algal pellet extracted with methanol (5 ml per g wet weight, for 2 h). The cells were then resedimented and the pellet was re-extracted in the same way with acetone and again with hexane.

Assays. The enzyme assays were done in microtitre plates and were based on the breakdown of a colorimetric substrate to produce a coloured product, enabling the reaction to be followed by measuring the absorbance over a period of time, at an appropriate wavelength, using an automatic ELISA plate reader. The enzyme and the test solution were preincubated to ensure maximum possible inhibition. Lack of colour development following the addition of substrate indicated enzyme-inhibitory activity. This system facilitated the simultaneous screening of many samples. Control samples of appropriate fresh media or methanol were included in all cases and inhibition of 50% or more was taken as a positive result. Duplicates of all samples were run. At the concentrations used here, the addition of methanol to the assay system did not appreciably affect the enzyme rate when compared to the rate obtained with fresh medium.

α -Glucosidase. α -D-Glucosidase [Sigma, type III, from yeast, suspension in 3.2 M-(NH₄)₂SO₄ solution] was dissolved in 0.1 M-potassium phosphate, 3.2 mM-MgCl₂, pH 6.8 (0.1 units ml⁻¹). The substrate was *p*-nitrophenyl α -D-glucopyranoside dissolved in 0.1 M-potassium phosphate, 3.2 mM-MgCl₂, pH 6.8. To each well of a microtitre plate was added 25 μ l 0.5 M-potassium phosphate, 16 mM-MgCl₂, pH 6.8; 100 μ l aqueous test solution, or 30 μ l methanolic (or acetone) extract and 70 μ l water; 50 μ l enzyme solution. This mixture was incubated at 37 °C for 30 min and then 50 μ l substrate was added. The absorbance of the mixture at 410 nm was measured at time 0 and after incubation at 37 °C, when significant colour change had taken place (approx. 0.4 A_{410} units h⁻¹).

α -Amylase. α -Amylase (Sigma, type I-A, DFP-treated, from porcine pancreas, twice crystallized suspension in 2.9 M-NaCl solution containing 3 mM-CaCl₂) was dissolved in 0.1 M-potassium phosphate, 0.05 M-NaCl, pH 7.1 (0.162 units ml⁻¹). The substrate was one substrate tablet from a Boehringer α -amylase PNP kit C-system, dissolved in 16 ml 0.1 M-potassium phosphate, 0.05 M-NaCl, pH 7.1. To each well of a microtitre plate was added: 25 μ l 0.5 M-potassium phosphate, 0.25 M-NaCl, pH 7.1; 100 μ l aqueous test solution, or 30 μ l methanolic (or acetone) extract and 70 μ l water; 50 μ l enzyme. This mixture was incubated at 37 °C for 30 min and then 50 μ l substrate was added. The absorbance of the mixture at 410 nm was read at time 0 and again after incubation at 37 °C, when significant colour change had taken place (approx. 0.4 A_{410} units h⁻¹).

β -Galactosidase. β -Galactosidase (Sigma, from *Escherichia coli*, suspension in 1.7 M-(NH₄)₂SO₄, 10 mM-Tris buffer salts and 1 mM-MgCl₂) was dissolved in 0.1 M-potassium phosphate, 16 mM- β -mercaptoethanol, 9 mM-MgCl₂, pH 7.3 (0.017 units ml⁻¹). The substrate was *o*-nitrophenyl β -D-galactopyranoside dissolved in 0.1 M-potassium phosphate pH 7.3 (1.5 mg ml⁻¹). To each well of a microtitre plate was added: 25 μ l 0.5 M potassium phosphate, 16 mM β -mercaptoethanol, 9 mM MgCl₂ pH 7.3; 100 μ l aqueous test solution, or 100 μ l methanolic (or acetone) extract; 50 μ l enzyme solution. This mixture was incubated at 37 °C for 30 min and then 50 μ l substrate was added. The absorbance of the reaction mixture at 410 nm was read at time 0 and again after incubation at 37 °C when significant colour change had taken place (approx. 0.4 A_{410} units h⁻¹).

RESULTS AND DISCUSSION

From a total of over 500 cultures tested, 38 species gave rise to inhibition in one of the three assays (Table 1). More strains inhibited α -glucosidase than α -amylase, and only one case of inhibition of β -galactosidase was observed. One might expect the lists of algae producing inhibitors to α -glucosidase and α -amylase to overlap to a large degree, as both enzymes hydrolyse 1,4- α -glucosidic linkages. However only three species appear on both lists. Evidently these enzymes, although hydrolysing the same bond by essentially the same mechanism, differ in their substrate specificity. It should also be noted that the enzymes used originated from different sources.

No one group of algae stands out as being particularly prolific in the production of inhibitory fractions, except perhaps the order Conjugales (of which a relatively small number were tested): culture extracts of six species were active against α -glucosidase, and two of these were active against α -amylase. Five different cultures of the cyanobacterial genus *Oscillatoria* produced inhibition of α -amylase; it is possible that these closely related species produced the same, or at least closely related, active compounds. Most of the species producing inhibitors are members of

Table 1. *Algae and cyanobacteria producing inhibitors to α -glucosidase, α -amylase or β -galactosidase*

CCAP strain no.*	Organism	Growth medium†	Solution containing inhibitor‡	Percentage inhibition§
(a) Strains producing inhibitors to α -glucosidase				
<i>Freshwater organisms</i>				
CHLOROPHYCEAE				
Chlorococcales:				
265/1	<i>Pseudochlorella antarctica</i>	C	M	55
276/4B	<i>Scenedesmus quadricauda</i>	C	M	60
276/20	<i>Scenedesmus subspicatus</i>	C	M	60
Ire	<i>Selenastrum minutum</i>	C	M	55
Conjugales:				
615/3	<i>Cylindrocystis brebisonii</i>	C	M	55
648/1A	<i>Mesotaenium caldariorum</i>	C	M	100
650/1	<i>Mougeotia</i> sp.	C	M	100
678/3	<i>Spirogyra varians</i>	C	M	100
698/2	<i>Zygnema cylindricum</i>	C	M	100
698/4	<i>Zygnema</i> sp.	C	M	90
Tetrasporales:				
31/1	<i>Gloeocystis maxima</i>	C	M	70
Volvocales:				
33/1	<i>Gloeomonas kupfferi</i>	C	M	55
34/7	<i>Haematococcus pluvialis</i>	C	M	55
CYANOBACTERIA				
Ire	<i>Anabaena spiroides</i>	B	Aq	100
Ire	<i>Anacystis nidulans</i>	B	M, Aq	100, 85
1414/1	<i>Coelosphaerium kuetzingianum</i>	B	A	85
1450/1	<i>Microcystis aeruginosa</i>	B	Aq	100
1453/26	<i>Nostoc</i> sp.	B	A	100
Ire	<i>Oscillatoria animalis</i>	B	Aq	100
<i>Marine organisms</i>				
CHLOROPHYCEAE				
Cladophorales:				
505/9	<i>Cladophora parriaudii</i>		Aq	80
Siphonales:				
702/1	<i>Acetabularia acetabulum</i>	S	Aq	100
Volvocales:				
80/1	<i>Asteromonas gracilis</i>	S	Aq	100
19/1	<i>Dunaliella parva</i>	S	Aq, M	100, 95
19/9	<i>Dunaliella parva</i>	S	Aq	95
11/34	<i>Dunaliella primolecta</i>	S	Aq	100
BACILLARIOPHYCEAE				
1034/1	<i>Gyrodinium spenceri</i>	S	Aq	90
1052/6	<i>Phaeodactylum tricornutum</i>	S	Aq	100
CHRYSTOPHYCEAE				
933/25	<i>Ochromonas villosa</i>	S	M	60
PRASINOPHYCEAE				
66/6	<i>Tetraselmis rubens</i>	S	Aq	100
PRYMNESIOPHYCEAE				
961/3	<i>Cricosphaera elongata</i>	S	Aq	100
RHODOPHYCEAE				
1360/9	<i>Audouinella parvula</i>	S	M	55
1360/11	<i>Audouinella</i> sp.		Aq, M	100, 50

(Table continued on next page)

Table 1. (*continued*)

CCAP strain no.*	Organism	Growth medium†	Solution containing inhibitor‡	Percentage inhibition§
(b) Strains producing inhibitors to α -amylase				
<i>Freshwater organisms</i>				
CHLOROPHYCEAE				
Conjugales:				
678/3	<i>Spirogyra varians</i>	C	M	55
698/4	<i>Zygnema</i> sp.	C	M	55
CYANOBACTERIA				
1403/13E	<i>Anabaena flos-aquae</i>	B	A, M	85, 90
1403/13F	<i>Anabaena flos-aquae</i>	B	A, M	85, 55
1403/13G	<i>Anabaena flos-aquae</i>	B	A, M	85, 95
1403/13H	<i>Anabaena flos-aquae</i>	B	M	70
1403/21	<i>Anabaena flos-aquae</i>	B	A, M	100, 95
1403/22	<i>Anabaena flos-aquae</i>	B	A, M	95, 80
1403/23	<i>Anabaena flos-aquae</i>	B	A, M	85, 90
1459/6	<i>Oscillatoria animalis</i>	B	A, M	90, 100
1459/11A	<i>Oscillatoria</i> sp.	B	M	75
1459/12A	<i>Oscillatoria redekei</i>	B	A, M	90, 70
1459/12B	<i>Oscillatoria redekei</i>	B	M	80
Ire	<i>Oscillatoria lutea</i>	B	A, M	95, 100
1470/2	<i>Schizothrix</i> sp.	B	A, M	60, 75
<i>Marine organisms</i>				
CYANOBACTERIA				
1446/4	<i>Lyngbya majuscula</i>	S	Aq	55
(c) Strains producing inhibitors to β -galactosidase				
<i>Marine organisms</i>				
CHLOROPHYCEAE				
Volvocales:				
11/86A	<i>Chlamydomonas plethora</i>	S	M	55

* Ire indicates cultures obtained from the Freshwater Biological Investigation Unit, Northern Ireland.

† B, medium BG-11; C, modified Chu 10; S, medium M11.

‡ A, acetone; Aq, aqueous; M, methanol.

§ Relative to the appropriate control.

the Chlorophyceae, but this is probably simply because the Chlorophyceae was the largest group of algae tested, and the list of inhibitor-producing organisms included representatives from many of the algal classes (Table 1).

It is recognized that the experimental conditions used would not lead to the detection of all the inhibitors that could be produced. At the time of harvest, the cultures may not have reached the point at which they begin to produce inhibitors, and as the inhibitors are presumably mainly a result of secondary metabolism, different media and growth conditions might well give rise to different secondary products. This is borne out by the fact that none of the freshwater eukaryotic algae, the only group to be grown in two different media, produced inhibitors in both media. In fact, activity was only found from cultures grown in modified Chu 10.

Several of the species listed in Table 1, e.g. *Anabaena flos-aquae* and *Microcystis aeruginosa*, produce toxins and antibacterial compounds (Gorham, 1964), but to our knowledge no one has reported the existence of glycosidase inhibitors from algae or cyanobacteria. Our results show that these organisms are worthy of further study as a source of potentially useful glycosidase inhibitors. We are currently investigating the nature of the compounds involved.

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