Purification and characterization of pentagalloylglucose, an α -glucosidase inhibitor/antibiotic from the freshwater green alga *Spirogyra varians*

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An α -glucosidase inhibitor/antibiotic was purified from the freshwater green alga *Spirogyra varians* and was determined to be the pentagalloylglucose 3-*O*-digalloyl-1,2,6-trigalloylglucose.

INTRODUCTION

Enzyme inhibitors have potential value in many areas of disease control and treatment. The control of kinetics of carbohydrate digestion and monosaccharide absorption could be of value in the prevention and control of conditions such as diabetes, obesity, hyperlipoproteinaemia and hyperlipidaemia, and in this respect inhibitors of glycosidases are of particular interest (Puls *et al.*, 1977).

Umezawa began screening actinomycetes for enzyme inhibitors in the 1960s, and since then many dozens have been described (Umezawa, 1972, 1982). Several interesting and potentially valuable inhibitors of glycosidases have been described, e.g. acarbose and nojirimycin (Truscheit *et al.*, 1981; Müller, 1985). The study of enzyme inhibitors from natural sources has, until recently, concentrated on bacteria and fungi. However, a recent large-scale screen for the presence of biologically active compounds identified enzyme-inhibitory activity from culture supernatants and organic-solvent extracts of a number of microalgae and cyanobacteria (Cannell *et al.*, 1987, 1988). These organisms have previously been little studied in this respect and are amenable to study under batch-culture conditions.

These activities included several inhibitors of the glycosidases (α -glucosidase, α -amylase and β -galactosidase). Several closely related freshwater green algae of the order Conjugales, namely *Spirogyra varians*, *Mougeotia* sp., *Zygnema cylindricum* and *Mesotaenium caldariorum* all produced inhibitors of α -glucosidase. One of these, an inhibitor of α -glucosidase, from the methanol extract of *Spirogyra varians*, was chosen for further study. This extract was also found to possess antibacterial activity.

MATERIALS AND METHODS

Algae

Cultures were obtained from the Culture Centre of Algae and Protozoa, Cambridge, U.K., and grown axenically in 1-litre conical flasks containing 600 ml of M4 medium (Asher & Spalding, 1982) at 23 °C with shaking at 100 rev./min with a 16 h-light/8 h-dark photoperiod. The biomass was harvested after 6 weeks growth

by filtering under vacuum through a Whatman 3MM filter.

Purification of inhibitor/antibiotic

The algal biomass was extracted with methanol (5 ml/g wet wt., shaking at 23 °C for 2 h). The extract was dried by vacuum centrifugation, redissolved in 0.05 M-phosphate buffer, pH 6.8, and extracted three times with equal volumes of diethyl ether and then butanol. The butanol extract was redissolved in methanol and chromatographed on a 10 cm $\times$ 0.75 cm column of Sephadex LH-20 developed with 95% (v/v) methanol (Fisons h.p.l.c. grade). The active fraction was purified by h.p.l.c. (h.p.l.c. was carried out on an LKB system incorporating a 2140 rapid spectral detector, 2150 h.p.l.c. pump, 2152 h.p.l.c. controller and an Olivetti M24 computer. Gel filtration was carried out using a Zorbax PSM 60S column with 95% h.p.l.c.-grade methanol at a flow rate of 0.5 ml/min as the eluting solvent).

Structure determination of inhibitor/antibiotic

Polyvinylpyrrolidone test. A standard method for the removal of tannins, and consequently a test for the presence of biologically active tannins, is the addition of polyvinylpyrrolidone (Loomis & Battaile, 1966). A 35 μ l portion of a crude methanol extract containing biological activity was mixed vigorously for 2 min with 0.1 g of polyvinylpyrrolidone (Polyclar AT) and incubated at 4 °C for 15 h. The mixture was briefly centrifuged at 5000 g for 2 min, and the supernatant was assayed for biological activity at a range of concentrations. Aliquots (35 μ l) of methanol treated in the same way as the extract were also assayed.

K₄Fe(CN)₆/FeCl₃ test (for reducing compounds, phenols, amines, thiosulphates and isothiocyanates). The inhibitor was spotted on to a silica-gel t.l.c. plate and sprayed with a mixture of equal values of aq. 1% K₄Fe(CN)₆ solution and aq. 2% (w/v) FeCl₃ solution.

M.s. This was carried out at the Medical Research Council Toxicology Unit.

(i) Fast atom bombardment. Samples were dissolved in methanol, and portions were applied to a glycerol

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matrix on a fast-atom-bombardment insertion probe. Spectra were determined on a VG 70-SEQ mass spectrometer operating in the positive- or negative-ion modes. Xenon atoms were used for sample bombardment with an atom gun (Ion Tech Ltd., Teddington, Middx., U.K.) current of 1 mA and a gun voltage of 8 kV. Ions were accelerated from the source region and mass-analysed after the electrostatic sector at a mass resolution of 1000 (5% valley) and a scan speed of 10 s/decade.

(ii) Electron impact and chemical ionization. A VG 70-SEQ mass spectrometer was operated with a source temperature of 220 °C and an accelerating voltage of 8 kV. For electron-impact operation the electron energy was 70 eV and the trap current 200 μ A; for chemical ionization the electron energy was 50 eV and the emission current 500 μ A. Isobutane was used as reagent gas for chemical ionization. Mass spectra were recorded at mass resolution 1500 and at a scan speed of 1 s/decade and were processed by a VG 11/250 data system. Ions were collected after the first two focusing sectors (electrostatic and magnetic) of the triple-sector instrument.

Biological (antibacterial) activity assay

Bacillus subtilis and *Micrococcus flavus* were grown overnight in nutrient broth (Oxoid no. 1) at 37 °C with shaking at 100 rev./min. Nutrient agar [0.28% (w/v), Oxoid no. 1] was dissolved in water by heating to boiling and then cooled to 50 °C. A 10 μ l portion of bacterial culture was pipetted into a 9 cm-diameter Petri plate and 30 ml of nutrient agar poured over this with thorough swirling to evenly seed the plate. Wells were bored using a 5 mm-diameter cork borer and filled with methanol extracts. These were preincubated at 4 °C for 2 h and then incubated at 37 °C for 24 h, after which time, confluent (or ideally semi-confluent) growth could be seen throughout the agar, except for clear zones around wells containing a bacterial inhibitor.

Enzyme-inhibition assays

α -Glucosidase. The enzyme solution consisted of α -D-glucosidase [0.01 ml; 5 units; Sigma Type III; from yeast; suspension in 3.2 M-(NH₄)₂SO₄] in 0.1 M-citrate/NaOH buffer, pH 6.9 (100 ml), containing 3.2 mM-MgCl₂. The substrate solution consisted of *p*-nitrophenyl α -D-glucopyranoside (35 mg) in citrate/NaOH buffer, pH 6.9 (100 ml), containing 3.2 mM-MgCl₂. In the assay, to each well of a microtitre plate were added 0.1 M-citrate/NaOH buffer, pH 6.9 (0.15 ml), 3.2 mM-MgCl₂, aqueous test solution (0.1 ml) or solvent extract (0.03 ml) and enzyme solution (0.05 ml). This mixture was preincubated at 37 °C for 30 min and then substrate solution was added (0.05 ml). The A_{410} of the mixture was measured at zero time and after incubation at 37 °C, when significant colour change had taken place (approx. 0.4 *A* unit/h).

Papain. The enzyme solution consisted of Papain (30 units; Sigma Type III; from *Papaya* latex) dissolved in 0.05 M-Tris/acetate buffer, pH 7.5 (10 ml), containing 5 mM-cysteine hydrochloride and 2 mM-EDTA. The substrate solution consisted of benzoylarginine 4-nitroanilide (43.4 mg; Sigma) dissolved in dimethyl sulphoxide (1 ml) and then made up to 100 ml with 0.05 M-Tris/acetate buffer, pH 7.5. For the assay, to each well of a microtitre plate were added 0.4 M-Tris/acetate buffer, pH 7.5 (0.05 ml), 5 mM-cysteine hydrochloride, 2 mM-EDTA,

aqueous test solution (0.1 ml) or methanol extract (0.05 ml) with water (0.05 ml) and enzyme solution (0.05 ml). This mixture was incubated at 37 °C for 30 min and then substrate solution (0.1 ml) was added. The absorbance was read at zero time and after incubation at 37 °C when significant colour change had taken place (approx. 0.25 *A* unit/h).

α -Amylase. The enzyme solution consisted of α -amylase (16.2 units; Sigma Type I-A; di-isopropyl phosphorofluoridate-treated; from porcine pancreas; twice-crystallized suspension in 2.9 M-NaCl/3 mM-CaCl₂) in 0.1 M-citrate/NaOH buffer, pH 7.1 (100 ml), containing 0.05 M-NaCl. The substrate solution consisted of one substrate tablet (*p*-nitrophenyl α -D-maltoheptaoside) from a Boehringer α -amylase PNP (*p*-nitrophenyl phosphatase) kit C-system, in 0.1 M-citrate/NaOH buffer, pH 7.1 (16 ml) containing 0.5 M-NaCl. For the assay, to each well of a microtitre plate were added 0.5 M-citrate/NaOH buffer (pH 7.1)/0.25 M-NaCl (0.025 ml), aqueous test solution (0.1 ml) or solvent extract (0.03 ml) with water (0.7 ml) and enzyme solution (0.05 ml). This mixture was preincubated at 37 °C for 30 min and then substrate solution (0.05 ml) was added. The A_{410} of the mixture was read at zero time and after incubation at 37 °C when significant colour change had taken place (approx. 0.4 *A* unit/h).

β -Galactosidase. The enzyme solution consisted of β -galactosidase [1.7 units; Sigma; from *Escherichia coli*; suspension in 1.7 M-(NH₄)₂SO₄/10 mM-Tris buffer salts/1 mM-MgCl₂] in 0.1 M-citrate/NaOH buffer, pH 7.3 (100 ml), containing 16 mM- β -mercaptoethanol and 9 mM-MgCl₂. The substrate solution consisted of *o*-nitrophenyl β -D-galactopyranoside (0.15 g) in 0.1 M-citrate/NaOH buffer, pH 7.3 (100 ml). For the assay, to each well of a microtitre plate were added 0.5 M-citrate/NaOH buffer, pH 7.3, containing 16 mM- β -mercaptoethanol and 9 mM-MgCl₂ (0.025 ml), test sample (0.1 ml) and enzyme solution (0.05 ml). This mixture was preincubated at 37 °C for 30 min and then substrate solution (0.05 ml) was added. The A_{410} of the reaction mixture was read at zero time and after incubation at 37 °C when significant colour change had taken place (approx. 0.4 *A* unit/h).

Time course of inhibitor production

Algal cultures were grown in 600 ml of M4 medium in 1-litre flasks with shaking at 100 rev./min. At alternate time intervals of 3 and 4 days, two aliquots of 10 ml were removed. One aliquot was filtered and dried to constant weight on filters previously dried to constant weight. The dry weight of the algal biomass was then calculated. The other aliquot was harvested by filtration and extracted with 5 ml of methanol. This extract was then assayed for α -glucosidase inhibition, in order to determine the volume of extract required to inhibit α -glucosidase by 50% under standard assay conditions. The biomass was multiplied by the above volume of extract to give an 'extract titre', a relative value inversely proportional to the amount of inhibitor per unit of biomass.

Kinetic studies of inhibition

The enzyme solution consisted of α -glucosidase as used for the assay for this enzyme described above. The inhibitor solution consisted of 0.04 mg of inhibitor/ml of

methanol. The substrate solution consisted of *p*-nitrophenyl α -D-glucopyranoside (8.7 mg) in 0.1 M-citrate buffer, pH 6.8, containing 3.2 mM-MgCl₂ (100 ml). This substrate concentration was equal to half the K_m for the enzyme. For the assay, enzyme solution (2 ml) and inhibitor solution (15 μ l) were mixed, and aliquots (0.2 ml) were removed at various time intervals over a 1 h period. These were assayed for enzyme activity by mixing with substrate solution (3 ml) and measuring the reaction rate by monitoring the increase in A_{410} over 2.5 min in Pye-Únicam SP.1800 spectrophotometer.

RESULTS AND DISCUSSION

Studies of α -glucosidase-inhibitor production over time showed that the amount of inhibitor produced per unit of biomass increased continuously and approximately linearly over the period of the growth curve (53 days) (Fig. 1). Kinetic studies showed that the inhibitor very probably acted irreversibly (Fig. 2).

The inhibitor, purified by solvent extraction, Sephadex LH-20 chromatography and h.p.l.c., reacted positively with K₄Fe(CN)₆/FeCl₃ and was inactivated upon addition of polyvinylpyrrolidone. It had absorption maxima at 280 nm and 216 nm, was water-soluble and was stable at pH 3 and 7 but not at pH 10.

M.s. results for the inhibitor

(i) **Negative-ion fast atom bombardment.** m/z (relative intensity), 940.3 [M (the molecular ion)–H][–] (2), 924.3 (M –OH)[–] (1), 787.9 [M –(HO)₃C₆H₂CO][–] (1), 770 [M –(HO)₃C₆H₂COO][–] (1.2), 771.0 (M –gallic acid)[–] (1.2), 770 (M –gallic acid–H)[–] (1.0), 617.8 (M –digallic acid–H)[–] (1.7), 601.8 [M –gallic acid–(HO)₃C₆COO][–] (1.2).

Fig. 1. Growth curve of *Spirogyra varians* (▲) and inhibitor production per unit of biomass (Δ)

The 'extract titre' is a relative value inversely proportional to the amount of inhibitor per unit of biomass (see the text).

Fig. 2. Effect on α -glucosidase activity of preincubation with inhibitor

(ii) **Positive-ion fast atom bombardment.** The only diagnostically significant ion was m/z 771 (M –gallic acid) (1.5), although ions were also detected extremely weakly in the molecular-ion region (941–942). Electron impact; m/z (relative intensity), 170 [gallic acid]⁺ (100), 153 [gallic acid–OH]⁺ (77).

M.s. results for pentagalloylglucose

(i) **Negative-ion fast atom bombardment.** m/z (relative intensity) 939.6 (M –H)[–] (10), 923.6 (M –OH)[–] (2), 787.5 [M –(HO)₃C₆CO][–] (3), 769.5 (M –gallic acid–H)[–] (3), 617.3 (M –digallic acid–H)[–] (2), 601.3 [M –gallic acid–(HO)₃C₆H₂COO][–] (3). The negative-ion fast-atom-bombardment spectrum of pentagalloylglucose was thus very similar to that of the inhibitor. (The slightly higher mass measurement values for the latter are presumably due to a mass calibration drift).

(ii) **Positive-ion fast atom bombardment.** m/z 770.8 [M –H–gallic acid]⁺ (1.5).

The results of the chemical tests suggested that the inhibitor could be a polyphenol, and the mass spectra were consistent with the inhibitor being a hydrolysable tannin of mass 940. This in turn suggested that the inhibitor might be pentagalloylglucose. At this point a review of the literature showed that tannins were first isolated from *Spirogyra* sp. by Nakabayashi and co-workers (Nakabayashi, 1954, 1955a,b; Nakabayashi & Hada, 1954a,b), who isolated pentagalloyl-D-glucose from *Spirogyra arcta*. Nishizawa et al. (1985) also isolated a number of galloylglucose molecules from *Spirogyra* sp. These ranged from tri- through to undeca-galloylglucose. T.l.c. of the inhibitor with a control sample of 1,2,3,4,6-penta-*O*-galloyl- β -glucose, in ethyl acetate/butanone/formic acid/water (5:3:1:1, by vol.), resulted in R_F values of 0.87 and 0.89 respectively. With the mass spectra, this suggested that the inhibitor was a pentagalloylglucose consisting of three monogallic acid units and one digallic acid. This was the gallotannin described by Nakabayashi and co-workers, and indeed 3-*O*-digalloyl-1,2,6-galloylglucose was the major form of pentagalloylglucose

Table 1. IC₅₀ (concentration causing 50% inhibition) of various forms of gallylglucose under standard α -glucosidase assay conditions

Compound	IC ₅₀
1,2,6-Tri- <i>O</i> -galloylglucose	6.8×10^{-6} M
1,2,3,6-Tetra- <i>O</i> -galloylglucose	3.4×10^{-6} M
1,2,3,4,6-Penta- <i>O</i> -galloylglucose	1.7×10^{-6} M
<i>Spirogyra</i> inhibitor	3.4×10^{-7} M

found by Nishizawa *et al.* (1985). Tests on the α -glucosidase-inhibitory activity of 1,2,6-tri-*O*-, 1,2,3,6-tetra-*O*-, 1,2,3,4,6-penta-*O*-galloylglucose and the purified inhibitor were carried out (Table 1). The digalloyl-containing form of pentagalloylglucose inhibited the enzyme with a potency approx. 5 times that of the 1,2,3,4,6-pentagalloylglucose; about 10 times the concentration of tetra- and about 20 times the concentration of tri-galloylglucose were required to produce the same degree of inhibition. It was not possible to test gallo-tannins possessing more than five gallic acid units, and therefore it was not possible to conclude whether increasing numbers of galloyl units led to increasing inhibitory activity or whether maximum activity was exhibited by pentagalloylglucose.

It has long been known that hydrolysable tannins are potent enzyme inhibitors, acting by their tendency to precipitate proteins. This precipitation is presumed to occur by the formation of hydrogen bonds between the hydroxy groups of the tannins and the peptide linkages of the proteins (Loomis & Battaile, 1966). Interestingly however, the material isolated in the present study demonstrated specific enzyme-inhibitory activity. The activity was specific for α -glucosidase and to a much lesser extent α -amylase; a 50-fold increase in inhibitor concentration was required to achieve the same level of inhibition in the α -amylase assay. The activity did not extend to the other carbohydrate-acting enzyme tested, namely β -galactosidase, or the other thiol enzyme tested, namely papain. This suggests that the α -glucosidic linkage, or the part of the enzyme's active site which recognizes the α -glucosidic linkage, is involved in determination of specificity. No activity was found against any other of the wide range of enzymes tested. Presumably the other enzymes of the screening programme were not inhibited because the tannins were not present in sufficiently high concentrations to significantly precipitate the enzymes. This suggests that the inhibition of α -glucosidase is effected by means other than enzyme precipitation. However, the inhibitor does appear to act irreversibly.

This compound was also found to be responsible for the antibacterial effects of the methanol extract of *Spirogyra varians*. Antimicrobial activity of a number of low-molecular-mass aromatic compounds of plant origin was recently described by Zemek *et al.* (1987). The active compounds included gallic acid, a major component of gallylglucose. The production of gallic acid by the metabolism of pentagalloylglucose possibly therefore accounts for the antibacterial activity of the galloylglucose isolated from *Spirogyra*. Interestingly, however, gallic acid, although a polyphenol, does not precipitate

proteins from aqueous solution and therefore does not exert antibacterial activity by this means. In contrast with green algae, phenolic compounds have been widely reported from brown and red algae and are largely responsible for the antibacterial effects of extracts of these algae. It is not clear whether these compounds serve any ecological or physiological role. The main function of phenolics in plants generally is probably as antimicrobial and anti-feeding agents. Vertesy *et al.* (1984) suggested that tendamistat, an α -amylase inhibitor isolated from *Streptomyces tendae*, might act as a regulator molecule of the organism's α -amylase. It is possible that this inhibitor, (and others identified from micro-algae), also act in this way. Many algae, particularly filamentous algae, a group that includes *Spirogyra*, produce large amounts of extracellular polysaccharides in the form of mucilage which enable the algae to adhere or aggregate. It is possible that the inhibitors serve to protect the mucilage from destruction by extracellular enzymes of potential predators. This theory is particularly appealing with regard to several cyanobacteria such as *Oscillatoria animalis* and *Anabaena flos-aquae*, which appear to be prolific producers of extracellular polysaccharides. Culture supernatants of these cyanobacteria were found to contain inhibitors of α -amylase (Cannell *et al.*, 1987).

Vicente *et al.* (1978) showed that a polyphenol, L-usnic acid, inactivates urease via the formation of aggregates. Aggregation takes place by the formation of L-alanyl-L-prolyl linkages, and are irreversible. Vicente (1985) suggested that production of phenolics by lichens could serve as a regulatory feedback-inhibition mechanism in the metabolism of urea by lichens. Usnic acid has also been shown to increase the permeability of *Spirogyra* cells, and this suggests that phenolics might also play a role in nutrient transfer in lichens (Vicente, 1985).

Polyphenolic compounds possessing 3,4-dihydroxy-phenol groups were found to inhibit α -acid oxidase in hop (*Humulus lupulus*), by a mechanism other than precipitation of the enzyme, and it has been proposed that some polyphenols act as specific antioxidants *in vivo*, thus providing a tolerance period during which time certain secondary metabolites might be sequestered (Williams & Menary, 1988).

The identification of pentagalloylglucose as an inhibitor of α -glucosidase, from *Spirogyra varians*, suggests that polyphenols, as well as acting as antimicrobial and anti-feeding agents, may play a greater role in the physiology of algae.

Studies on the α -glucosidase inhibitors identified from the methanol extracts of related algae (*Zygnema cylindricum*, *Mougeotia* sp. and *Mesotaenium caldararum*) have indicated that these are not tannins.

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