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Isolation and Partial Purification of a Red Tide (*Gymnodinium breve*) Cytolytic Factor(s) from Cultures of *Gomphosphaeria aponina*

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Material(s) that is cytolytically active toward the red tide organism *Gymnodinium breve* has been isolated from the blue-green alga *Gomphosphaeria aponina*. A cytolytic assay was developed and is described. Optimum yields of cytolytic material(s) were obtained by extraction of cultures of *G. aponina* with chloroform at neutral pH. Purification of the cytolytic material(s) was attempted using chloroform–methanol mixtures with best results obtained with elution on activated alumina (WB-2) columns, silica thin-layer chromatography, and silica high-performance liquid chromatography. Considerable losses of activity and specific activity were noted during purification, but the activity and specific activity could be restored by pooling fractions obtained in the separations. Mass spectral analysis of a purified fraction with maximum activity indicated a significant fragment with probable formula of $C_{29}H_{49}O$.

Enthusiasm for marine aquaculture in the near-shore waters of the Gulf of Mexico is undoubtedly mitigated by a number of factors, including the absence of agents suitable for managing periodic red tides. In Florida's coastal waters, for example, red tides are associated with periodic, sporadic blooms of the unarmored dinoflagellate, *Gymnodinium breve* (cf. Martin and Martin, 1976a), which result in massive mortalities of marine animals. The exact cost of these outbreaks is uncertain, but the estimated cost of the 1971 outbreak that lasted for 3 summer months and covered a coastal area of seven counties was $17 000 000 (Habas and Gilbert, 1974).

Some screening studies have been concerned with chemicals that would be effective in managing *G. breve*. Marvin and Proctor (1964) examined 4800 chemicals for this purpose, though expense and other considerations apparently limited follow-up studies.

We believe that isolation of cytolytic material(s) (provisionally called "aponin") from cultures of the blue-green alga, *Gomphosphaeria aponina*, represents a potentially promising approach to management of red tides (Kutt and Martin, 1975; Martin and Martin, 1976a). First, aponin (and the associated organism) was isolated from a 1973 red-tide outbreak (Kutt and Martin, 1974, 1975). In addition, it has been demonstrated (McCoy and Martin, 1977) that crude aponin was not ichthyotoxic to test fish, *Poecilia sphenops*, and, in fact, the ichthyotoxicity of *G. breve* cultures was mitigated at certain concentrations of crude aponin. More recently, it was demonstrated that concentrations of aponin that were effectively cytolytic toward *G. breve* cultures did not kill brine shrimp, *Artemia salina* (Eng-Wilmot and Martin, 1978b).

The present report describes the optimization of aponin isolation, development of an effective cytolytic bioassay, and fractionation of the crude isolates into two chromatographically pure biologically active fractions.

EXPERIMENTAL SECTION

Organism Source and Culture. Unialgal cultures of *Gymnodinium breve* were obtained through the courtesy of W. B. Wilson and S. M. Ray (Texas A&M Marine Institute, Galveston) and were maintained as axenic stock cultures in either artificial sea water or natural aged sea water, salinity, *S*, 33 ppt enriched with modified B-5 supplements as described by Brydon and co-workers (1971). *Gomphosphaeria aponia* was isolated from environmental samples (Kutt and Martin, 1975) and identified by Dr. C. J. Dawes (cf. Dawes, 1974). Unialgal bacterial-free stock cultures were maintained in suitably enriched artificial sea water, *S*, 28 ppt (Eng-Wilmot and Martin, 1978a). Large-scale semicontinuous cultures of *G. aponina*, grown under optimized culture conditions (Eng-Wilmot and Martin, 1977b), served as the organism source in these studies.

The microorganisms were enumerated with a Coulter Counter (Model B) equipped with a C-1000 Channelyzer and 100-μm aperture, with electronic and threshold set-

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tings as previously described (Eng-Wilmot et al., 1977a).

Isolation Procedure. Cultures of *G. aponina* were grown in 30–35-L quantities and were harvested shortly after stationary phase populations of 50–75 000 cells/mL were attained. Cells were harvested by continuous centrifugation using a DuPont Sorvall SS-3 centrifuge, a SS-34 head, and the Szent-Gyorgi and Blum apparatus, at 12 000*g*, and a flow rate of 100 mL/min. The cell-free centrifugate was extracted for 24 h (1 h of mixing) under neutral conditions (pH 7.2–8.1) with 50 mL/L of redistilled chloroform. The aqueous phase was decanted and the remaining sea water separated from the organic layer using a large cylindrical separatory funnel. The chloroform layer was then evaporated to dryness by rotary evaporation (50 °C, 75 torr), and the residues redissolved in a known volume of chloroform (4–10 mL). This crude preparation, termed "aponin" (Kutt and Martin, 1975) was assayed for cytolytic activity, and subsequent purification was attempted using the procedures described below.

Cytolytic Bioassay. A sensitive assay was developed for the determination of relative concentrations of aponin in crude and partially purified samples, based on its cytolytic action toward *G. breve*. Aliquots of crude or partially purified samples were pipetted into 15-mL Pyrex tubes, and the chloroform was evaporated in an evacuated oven (41 °C, 75 torr). To each assay tube was carefully added 12 mL of a well-mixed sample of *G. breve* culture in log-phase growth. Optimum results were obtained by mixing 500–750 mL of culture in an illuminated New Brunswick Scientific G-62 Gyrotory water shaker bath for 30 min at 120 rpm. Cultures having 1500–3000 cells/mL were routinely used. Treated assay tubes were gently mixed using a Labquake shaker for 1 h. After 20 h of incubation, at constant illumination (2000 lx, 40-W cool-white fluorescent lamps) and ambient temperature (25 ± 1 °C), the remaining *G. breve* cells were enumerated. Cell viability was assessed using a microscope (40×). All assays were run in triplicate, with redistilled chloroform used as the solvent control.

Thin-Layer Chromatography (TLC). Several types of thin-layer plates were used in the separation and purification of aponin. Commercially prepared thin layers of alumina (Eastman Chromagram sheet 6063), silica (Eastman Chromagram sheet 6060), and silica gel F (ICN Pharmaceuticals GmbH and Co., Eschwege, West Germany) were activated by heating 30 min, 50 °C, 75 torr) prior to use (Stahl, 1969). Plates were spotted with 25-μL samples and chromatographed in either the so-called sandwich plate or S-chamber (Eastman Chromagram chamber plate set) (Stahl 1969), or in a Gelman saturation chamber (Model 51325-1) equipped with a solvent saturation pad which was preequilibrated with solvent. Analytical or spectral grade elution solvents were freshly distilled in all-glass stills prior to use.

Identification of spots on chromatograms was accomplished by spraying with an atomizer containing a 50% sulfuric acid solution saturated with potassium dichromate, followed by heating for 10 min at 110 °C, or by fluorescence using a UVP, Inc. ultraviolet lamp. In addition, specific reagents for chemical groups were used and are described in the Results.

Column Chromatography. Separation and partial purification of aponin was achieved to various extents using column chromatography: silica gel, 100–200 mesh (Matheson, Coleman and Bell, SX144-7), and washed three times with 10% (v/v) CH_3OH in $CHCl_3$, and activated for 24 h at 100 °C (Stahl, 1969). Chromatographic alumina (basic WB-2 Sigma), as activity grade one (Brockman and Schodder, 1941), was pretreated with several column volumes of the eluting solvent. Gel filtration, using Sephadex LH-20-100 (Sigma) was also attempted. Gels were prepared in the same manner as the alumina. Glass columns, generally 1 × 30 cm or 1.5 × 50 cm, were used and eluted at a rate of 8–16 mL/min with a linear gradient of methanolic chloroform (0–40%, v/v). The fraction volume was 8 mL.

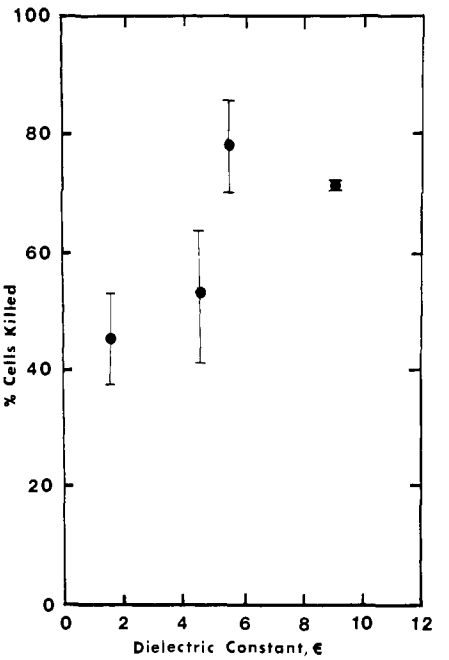

Figure 1. Effectiveness of various solvents in the extraction of "aponin", as a plot of the percentage (%) of *Gymnodinium breve* cells killed as a function of the dielectric constant (ϵ) of the solvent (ϵ increases from n-C_6H_{14} to CH_2Cl_2 to $CHCl_3$ to $(C_2H_5)_2O$).

Differential absorbance (A_{265}) was determined using a Beckman DB-GT spectrophotometer, and biological activity of each fraction was assayed.

High-Performance Liquid Chromatography (LC). Crude aponin was also separated with LC techniques, using a Waters Associates chromatography pump, M-6000 (700 psi; 1.5 mL/min), a 25-cm deactivated Partisil 10 silica gel column, and a linear gradient of CH_3OH in $CHCl_3$. Elution was monitored at 254 nm, using a Beckman DB-GT spectrophotometer and 30-μL flow cell, with $CHCl_3$ in the reference cell. Fractions were collected and stored in sealed ampules in a freezer until assayed.

Mass Spectrometry. High-resolution mass spectral analysis of two samples of the chromatographically pure fraction of major activity ($aponin_1$) was performed by Dr. Donald Hunt (University of Virginia, Charlottesville) using a Finnigan Model 3200 quadrupole mass spectrometer equipped with a chemical ionization source. Spectra were recorded every 4 s, and the results were subjected to computer analysis.

RESULTS

Optimization of Extraction. Previous investigations (Kutt and Martin, 1975; Martin and Martin, 1976b) of the cytolytic activity of cultures of *G. aponina* did not consider optimization of the yield of active material. The present study has examined this in terms of the choice of solvent and method of extraction, while the distribution of active material in different fractions of the cultures has been reviewed elsewhere (McCoy and Martin, 1978).

Four solvents (hexane, dichloromethane, chloroform, and diethyl ether) were examined for their effectiveness in extracting cytolytically active material. The results are

presented in Figure 1, where effectiveness, as percent of *G. breve* cells destroyed, is presented as a function of the dielectric constant of the solvent. No statistically significant difference of extraction efficiency was observed between diethyl ether and chloroform; chloroform was selected as the extraction solvent.

Several methods of extraction were investigated. Continuous cell centrifugation, followed by chloroform extraction (without addition of acid or base) of the cell-free centrifugate was commonly used. Most of the activity (>60%) was found to be associated with the cell-free medium. In addition, using this procedure, extracted samples were less contaminated with pigments than with whole cultures extraction. Present and previous (Martin and Martin, 1976b) studies showed the stability of cytolytic material in neutral or acidic medium, but activity was lost at higher pH (pH >9).

Bioassay of Cytolytic Activity. Several details must be considered in order to have good precision in the bioassay. First, to obtain a uniform stock culture, cells in log-growth phase were mixed in a gyrotory shaker bath. If the fraction of cells destroyed on shaking for 20 min exceeded 10%, the stock culture was not used in the bioassay. Secondly, optimum precision was 3% when 1500–3000 cells/mL were used (cf. Eng-Wilmot et al., 1977a). Third, the length of interaction (20 h) was a reasonable compromise.

Activity was expressed as cytolytic units (or aponin units), defined as that amount of cytolytic material required to lyse 50% of the *G. breve* cells under defined conditions (12 mL of culture, 2000 cells/mL, 20 h incubation at 25 °C). The aponin unit is essentially an ED_{50} dose, though it is expressed in terms of the amount of material required to lyse half of the cells in a population of 24 000 cells, e.g., one aponin unit represents 24 000 ED_{50} units.

Based upon this definition, the number of aponin units in a given sample was calculated:

$$\text{units} = (N - n)/1000 \quad (1)$$

Here, N represents the concentration of cells in the control sample after 20 h incubation [which was generally not significantly different (1–3%) from the concentration of cells initially], and n is the concentration of cells in the test culture at the end of the incubation period. The optimum number of units per sample ranged from 0.4–1.8 (vide infra). Then, the concentration of cytolytic material in a given extract could be evaluated:

$$\text{aponin units/mL of extract} = \frac{(N - n)}{1000(\text{mL of extract used})} \quad (2)$$

Implicit in the application of eq 1 or 2 is the assumption of a linear relationship between number of aponin units and the number of cells lysed. The relationship is actually sigmoidal (specifically for a plot of log units as a function of the log milliliter of extract). In the region selected (0.4–1.8 units), however, the relationship was sufficiently linear for precise estimation of the cytolytic activity.

Results for cytolytic activity, expressed as aponin units, were obtained by expressing the results in terms of probits (Finney, 1962), which converts a sigmoidal relationship (percentage of cells lysed vs. concentration of lysing agent) to a linear one (probits vs. concentration of lysing agent): 50% lysis corresponds to a probit of 5 (Figure 2).

Finally, the cytolytic activity was also related to the carbon content using the chromic acid oxidation method of Strickland and Parsons (1965), with glucose as the standard. In this way, the activity was expressed as aponin units and as specific activity units/milligram of oxidizable carbon.

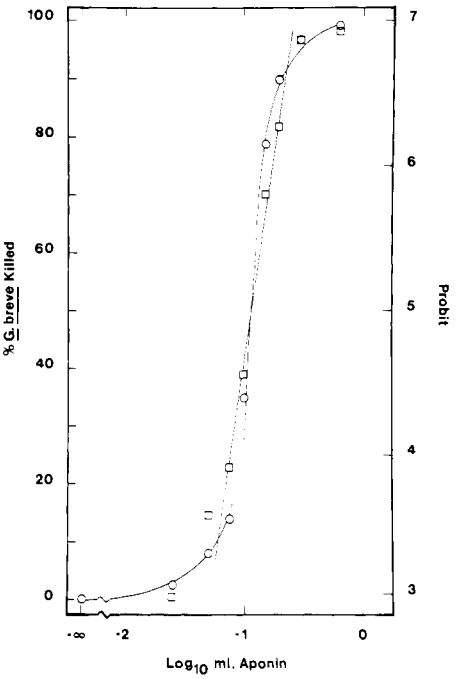

Figure 2. Dose–response curve for the cytolysis of *Gymnodinium breve* cells by aponin, represented as a plot of the variation in % cells lysed (○) and the probit of % cells lysed (□) as a function of log mL of aponin applied.

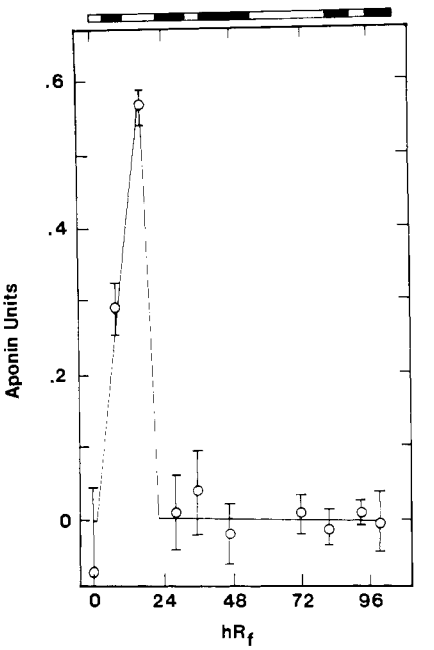

Figure 3. Separation of crude aponin by $CHCl_3$ elution of samples on TLC (silica gel) in an S-chamber, plotted as units of activity as a function of hR_f ($100 \times R_f$).

Thin-Layer and Column Chromatography. Twelve solvent systems were examined using TLC. Optimum separation (largest number of spots) was obtained using chloroform, activated silica gel, and an S-chamber. The TLC plate was separated into ten fractions (Figure 3 as the upper coordinate), and each was extracted with 20% (v/v) methanolic chloroform. Cytolytic activity as a function of hR_f ($=R_f \times 100$) showed a single sharp peak (fraction 3), with a significant activity in fraction 2.

Table I. Summary of Results of the Purification Scheme for Aponin$_1$ from Crude Aponin Preparations, Using Preparative Silica Thin-Layer and Activated Alumina Chromatography

preparative step	aponin[a] units	oxidizable[b] carbon, mg	units/mg of oxidizable carbon	relative[c] purification	% recov.	no.[d] of TLC spots
crude extract I	1520	167 (220)	9.1	1.00		>11
crude extract II	570	57 (74)	10.0	1.10		⩾9
column I (alumina)	37	34 (44)	1.1	0.12	2	7
column II (alumina)	18	6 (8)	3.0	0.33	49	2
preparative thin-layer chromatography (silica)	10	0.7 (0.9)	14.3	1.60	56	1

[a] Aponin unit = $(N - n)/1000$. [b] Oxidizable carbon relative to glucose (Strickland and Parsons, 1965). In parentheses, weight (in mg) of crude sample equivalent to 1 mg of oxidizable carbon. [c] Relative purification = (units/mg)/(units/mg preceding step). [d] Number of TLC spots (silica gel, $CHCl_3$ elution) observed in fraction containing the cytolytic activity.

Table II. Summary of Two Separations of Crude Aponin on Activated Alumina Columns[a]

sample	aponin units	oxidizable[b] carbon, mg	units/mg of oxidizable carbon	% recov.	rel. activity
		Run 1			
crude aponin	508	32.3	15.7		1.0
column[a] eluant	290	10.9	26.7	57	1.7
		Run 2			
crude aponin	1019	36.8	27.7		1.00
fraction I[c]	80	20.6	3.9	7.9	0.14
fraction II[c]	16	13.4	1.2	1.5	0.42
fractions[d] I and II	994	34.0	29.0	97.0	1.10
fraction[e] IA	978	20.6	48.0	96.0	1.70

[a] Fractions were collected from a 1-by-30-cm activated alumina (WB-2) column. [b] Oxidizable carbon relative to glucose (Strickland and Parsons, 1965). [c] Fraction I was a pooled concentrate of active fractions eluted. Fraction II was the pooled concentrate of all other fractions. [d] Assays were carried out with 0.5 mL of fraction II and various concentrations of fraction I. [e] Fraction IA was taken from *d* but the contribution of fraction II to the oxidizable carbon and activity was substracted from mixture values.

Two other points concerning preparative TLC deserve mention. First, fraction 3 actually consisted of three components (fluorescence and chromic acid oxidation) that could not be effectively separated because of their close proximity (hR_f 10.8, 15.7, 16.9). Secondly, the activity of fractions 2 and 3 was about 50 units or only about 10% of the initial activity. The apparent loss of activity is obviously significant and is considered subsequently.

A crude preparation was chromatographed on activated alumina and the fractions showing maximum activity were pooled, concentrated, and rechromatographed (Figure 4). Two active components (fractions 37–39 and 41–42) were obtained. The first appeared to be the major component (aponin$_1$) and was obtained in greater amounts in this subsequent experiments; the second fraction was obtained in lesser or slight amounts. Aponin$_1$ appeared to be chromatographically pure, based upon the observation of a single spot (with chromic acid) on a TLC plate (hR_f 17–20 on activated silica gel). The total activity for two-stage separation was about 15% of the initial activity.

A large-scale preparation (190 L) yielded about 2100 units of aponin from a whole culture extraction (two stages, crude extract I and II, Table I). Two observations are significant. First, a chromatographically pure fraction (aponin$_1$, hR_f 17–20) was obtained at the fourth stage. Second, the decline in activity was substantial. The crude material contained 2100 units, and the active fractions eluted from the column contained 37 units. Worse yet, the specific activity, expressed as units of aponin/milligram of oxidizable carbon, decreased from 9–10 to 1–3 (Table I). Third, the activity could be restored by recombining fractions (cf. 28 line 3 vs. 29 units/mg of oxidizable carbon, line 6, Table II).

A second series of experiments (summarized in Table II) repeated the large-scale separation, using about 1000

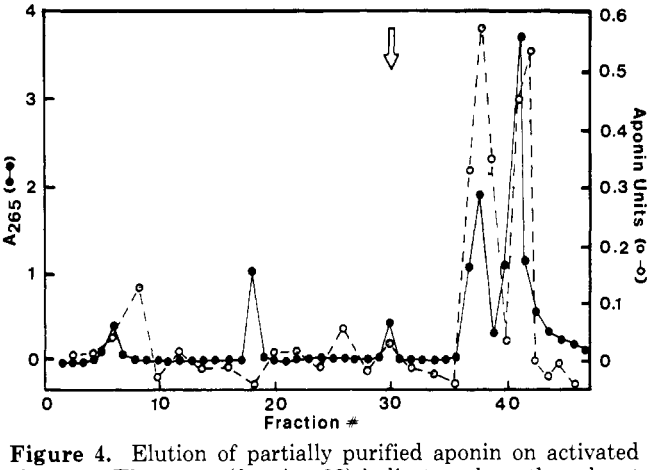

Figure 4. Elution of partially purified aponin on activated alumina. The arrow (fraction 28) indicates where the solvent system was changed from $CHCl_3$ to 10% (v/v) CH_3OH–$CHCl_3$.

units, in an effort to check for synergism. The fractions with significant activity were recombined, and it was possible to restore the specific activity. For example, the initial specific activity was 28 units/mg of oxidizable carbon for the crude material, 3.9 and 1.2 for active fractions (aponin$_1$ and aponin$_2$), and 29 for recombination. In fact, an enhancement of specific activity (to 48 units/mg) can be ascertained, if a correction is made for the contribution of oxidizable carbon and activity of second fraction to the mixture. This experiment indicated a synergistic effect but it did not serve to specify which of the subfractions might be involved.

High-Performance Liquid Chromatography. Samples (10 μL, 20.5 units) were separated with moderate resolution into six to ten fractions (Figure 5). Chroma-

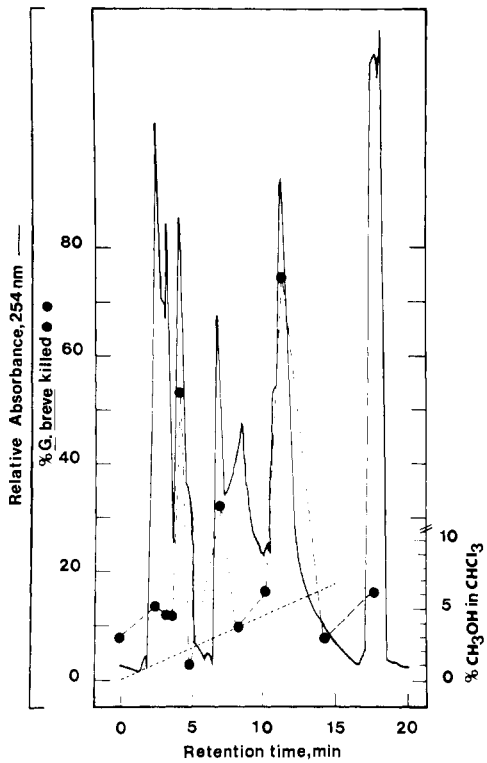

Figure 5. Representative LC elution and cytolytic activity profiles of crude aponin on Partisil 10, using linear gradients of CH_3OH in $CHCl_3$ (dotted line). After 15 min, elution was with 100% CH_3OH. Fractions exhibiting significant activity were $aponin_1$ (hR_f 16) and $aponin_2$ (hR_f 90.2).

tographic purity was assessed on activated silica gel TLC, in a S-chamber, with $CHCl_3$ as the resolving eluant. Two chromatographically pure fractions (hR_f 16.0 and 90.2) were identified that exhibited a majority (82%) of the recovered activity (151% recovery, determined as a summation of activities of all fractions). The two active fractions were classified as the major (hR_f 16, $aponin_1$) and minor (hR_f 90.2, $aponin_2$ activity components. $Aponin_1$ exhibited about 50% of the summed total of activities of all separated fractions, with $aponin_2$ possessing about 35%. Activity associated with the other fractions was negligible and varied up to about 10% of the total. All fractions were colorless (except $aponin_2$ which was slightly golden colored); all fluoresced on irradiation of TLC plates.

Preliminary Characterization. Spectroscopic and chemical studies of purified samples of the major activity component, $aponin_1$, yielded limited, but valuable information about its structural functionalities and properties.

Typical spectroscopic techniques (UV, IR, NMR) indicated several important structural features. The UV spectrum, obtained with a Cary-14 spectrophotometer of a sample in 16% (v/v) isopropyl alcohol–hexane, had little absorption above 210 nm, although a small shoulder was observed at 265 nm, which indicated $aponin_1$ had neither aromatic, nor conjugated C=C systems, and lacked the bromo, iodo, and nitro chromophores. The IR spectrum (Perkin-Elmer 225 grating infrared) of a sample evaporated onto a KBr pellet confirmed this lack of aromaticity, conjugation, and unsaturation, but revealed significant carbonyl (1720 cm^{-1}) and hydrogen bonding (OH or NH, 3400 cm^{-1}) stretching vibrations. Attempts to obtain an NMR spectrum, using the Varian A-60 and Varian C-1041 signal-averaging computer, resulted in a spectrum of unsatisfactory quality, owing to problems with the system and sample concentration, but did show a broad absorption between τ 6 and 8. Qualitative examination of these spectral characteristics, along with chemical information obtained from specific TLC tests described below, suggested that $aponin_1$ belonged to one of several possible classes of natural products, which included sterols.

Spot tests for several chemical groups were performed on thin-layer chromatographed samples. A positive 2,4-dinitrophenylhydrazine (2,4-DNP) test was observed with spots corresponding to hR_f (=16) values of $aponin_1$, using 2 M HCl saturated with 2,4-DNP. Anisaldehyde was the positive control (Stahl, 1969). This confirmed the presence of the carbonyl group observed in the IR spectrum. Treating plates with ninhydrin gave negative results and indicated the absence of α-amino group. Sensitive spot tests for steroids, vitamins, lipids, and carbohydrates gave positive results. Treating the TLC plates with 20% antimony trichloride in $CHCl_3$ (Antony and Beher, 1964) gave a positive test, indicative of steroids, vitamins, or lipids. $Aponin_1$ gave a positive test at an hR_f of 17.5, and cholesterol, the positive control, had an hR_f of 16.1. Spraying plates with methanolic anisaldehyde in H_2SO_4 (Stahl, 1969) was also positive. The Lieberman-Burchard test for sterols (cf. Cook, 1958) using acetic anhydride and concentrated H_2SO_4 also gave a positive result with $aponin_1$, as did β-sitosterol and stigmasterol, the positive controls.

High Resolution of Mass Spectra of $Aponin_1$. Large-scale purifications of $aponin_1$ provided sufficient material for a high-resolution mass spectral analysis, as described earlier. The distribution of m/e values was extremely complex, and as yet, complete fragmentation patterns have not been worked out. Positive ions having m/e values less than 275 appeared in all of the spectra, and significant amounts of material having m/e values greater than 275 appeared after 160 s. Fragments with m/e values of 397 and 413 were observed with relative abundancies of 94.3 and 41.1, respectively, at 172 s.

Computer analysis of the m/e peak at 397 (exact mass 397.3834 and 397.3852 for two different preparations) predicted a formula $C_{29}H_{49}$. Analysis of molecular ion, M^+ peak at 413 (413.3793 and 413.3783 for the two samples), yielded a predicted formula of $C_{29}H_{49}O$. The formulas and high relative abundancies of the two fragments were consistent with a C-29 steroid or steroid-like compound (Dr. Donald F. Hunt, personal communication) and were compatible with chemical and spectral data obtained using other techniques.

DISCUSSION

Earlier studies clearly demonstrated the association of the cytolytic factor, "aponin", with *Gomphosphaeria aponina* cells (Kutt and Martin, 1975; Martin and Martin, 1976b; McCoy and Martin, 1977). Recently, McCoy and Martin (1978) reported that greater than 60% of cytolytic activity was associated with the cell-free supernate. This indicated that the material was primarily exotoxic, being elaborated during the growth cycle of the organism, as observed, in growth-phase biosynthesis studies conducted (Eng-Wilmot and Martin, unpublished data). Consequently, isolation of bioactive materials was limited primarily to the cell-free supernate. Optimal isolation efficiency was observed under neutral extraction conditions with organic solvents exhibiting limited polarity, e.g., chloroform or diethyl ether. More polar solvents, such as ethanol, were unsuitable for reasons of miscibility in water, reduced yields, and the additional purification steps required for removal of soluble pigments and membrane components.

Although exact quantitation of the cytolytic bioassay of "aponin" has not yet been satisfactorily achieved, several significant features of the present procedure deserve at-

tention. Homogeneity of the *G. breve* test cultures (mixing) is essential in such a procedure, particularly if good precision is to be achieved, although continued mixing resulted in the changes in *G. breve* morphology, also noted by Baldridge and Klein (1974). Moreover, this provided a means of assessing cell fragility of the test cultures, and thus alleviated problems of variability of cytolytic response of different stock cultures. Secondly, preliminary cytolysis kinetic studies indicated that most of the cell degradation occurred within 20 h, with about 40–60% of the total effect taking place within the first 3–4 h of the assay.

Finally, the solubility of aponin requires comment. Judging from the polarity of extraction and purification solvents, the bioactive components isolated should exhibit considerable nonpolar character. There is reason to suspect insolubility of the purified compounds in aqueous solutions, although it is reasonable that they must exhibit some "intrinsic" water solubility. Residues from evaporation of solvents from crude preparations do exhibit some insolubility, particularly at dosages greater than 4 units. Insolubility, however, is alleviated to some degree by perhaps one of several factors. If the active components were weakly associated with water-soluble pigment, protein, or lipid materials, their aqueous solubility might be significantly enhanced, in a fashion similar to that described by Westphal (1961) (cf. Villee and Engel, 1961). Alternately, the presence of a polar moiety attached to the active molecule, as in steroid or terpenoid saponins (a sugar or polysaccharide attached through a glycosidic linkage to an aglycon) would be sufficient to enact water solubility (Heftman and Mosettig, 1960).

Chemical and structural examination of aponin$_1$, the major activity component, has yielded limited, but useful information about the nature of this compound. Chemical tests performed on TLC separated components indicated that the major active principle contained structural features resembling those of a steroid. A 2,4-DNP derivative was also observed, indicative of a ketonic carbonyl, which was also observed in the IR spectrum, although the $\pi \rightarrow \pi^*$ transition for an isolated carbonyl (ca. 280 nm) was not observed in the UV region. On the other hand, a positive Lieberman-Burchard test, indicative of unsaturated sterols, was obtained. Undoubtedly, some type of reduction or keto–enol rearrangement observed with certain types of basic alumina chromatography of steroids (cf. Posner et al., 1977) has taken place. Additional information about aponin$_1$ was obtained from mass spectral analysis. The molecular ion (M^+) was observed at m/e 413, and identical spectra were obtained for two different determinations. The computer predicted formula for M^+ was $C_{29}H_{49}O$, with five degrees of unsaturation. In addition, a major M – 16 peak was observed at m/e 397 (397.383 and 397.383 were observed; calculated values were 397.380 and 397.378, respectfully). Obviously, a number of compounds are possible, though chemical and spectral information are consistent with a sterol or steroid-like compound. Whether or not aponin$_1$ is a free sterol or a plant glycoside (steroid or terpenoid saponin) remains unresolved. If it were the latter, however, then significant degradation of the parent compound has taken place during the course of purification to give the results obtained.

In summary, it appears reasonable to suggest the logical place to seek a natural control agent for *Gymnodinium breve* is the near-shore environment. The present study has indicated one such agent may be aponin$_1$, which is effective in the laboratory setting. Knowing the properties of aponin$_1$ would permit field analyses for aponin$_1$ or similar agents, either through direct analysis (MS–GC) and/or through cytolytic assay in comparison with authentic samples. Such studies should provide answers to intriguing questions including those concerning the extent of natural control of *Gymnodinium breve*, whether species such as *G. aponina* may play a significant role in the natural protection of marine animals from red tide blooms, whether natural products such as aponin$_1$ could assist efforts to extend aquaculture in near-shore waters, and whether aponin$_1$ or similar agents are involved in the disappearance of *G. breve* at the bloom.

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