A New Procedure for the Isolation of Anti-HIV Compounds (Polysaccharides and Polyphenols) from the Marine Alga Fucus vesiculosus

Anne Béress, Otmar Wassermann, Thomas Bruhn, László Béress, Edmundo N. Kraiselburd, Luz Virginia Gonzalez, Gladys E. de Motta, and Pedro I. Chavez

J. Nat. Prod., **1993**, 56 (4), 478-488• DOI: 10.1021/np50094a005 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 4, pp. 478-488, April 1993

A NEW PROCEDURE FOR THE ISOLATION OF ANTI-HIV COMPOUNDS (POLYSACCHARIDES AND POLYPHENOLS) FROM THE MARINE ALGA *FUCUS VESICULOSUS*

ANNE BÉRESS, OTMAR WASSERMANN, THOMAS BRUHN, LÁSZLÓ BÉRESS,

Institute of Toxicology, Christian Albrechts-Universität, Brunswiker Str. 10, D-2300 Kiel, Germany

EDMUNDO N. KRAISELBURD*, LUZ VIRGINIA GONZALEZ,

Department of Microbiology, University of Puerto Rico, School of Medicine, M.R.R. PO Box 365067, San Juan, Puerto Rico

GLADYS E. DE MOTTA,

Institute of Neurobiology, University of Puerto Rico, School of Medicine, Blvd. del Valle 201, San Juan, Puerto Rico

and PEDRO I. CHAVEZ

School of Pharmacy, University of Puerto Rico, GPO Box 5067, San Juan, Puerto Rico

ABSTRACT.—Anti-HIV-active polysaccharides and polyphenols were isolated from the brown seaweed *Fucus vesiculosus* by hot H_2O extraction of both the intact and the homogenized algae. This was followed by XAD_2 chromatography and by sequential precipitation of the non-adsorbed compounds with glacial HOAc and thereafter with EtOH. The precipitate was solubilized, dialyzed against distilled H_2O, and chromatographed on SP-Sephadex C25 and on QAE-Sephadex A25. This was followed by gel filtration on Sephadex G50 and Sephadex G100 and finally by hplc on a Shodex Ionpak S-804 column. For comparison, the commercial product fucoidan, a sulfated algal polysaccharide, was also further purified by the chromatographic techniques mentioned above. The isolated freeze-dried fractions obtained by these procedures were tested for inhibition of both HIV-induced syncytium formation and HIV reverse transcriptase enzyme activity. Some of these fractions inhibited both of these activities at concentrations that were not cytotoxic.

The brown seaweed *Fucus vesiculosus* L. (Fucales) is a common littoral alga of the coasts of the Northern Atlantic, the Pacific Ocean, and the Baltic Sea. During studies to isolate antiviral compounds from marine organisms, the H_2O-soluble extracts from this alga inhibited the activity of the human immunodeficiency virus (HIV) reverse transcriptase (RT) enzyme as well as HIV-induced syncytium formation. Chemical composition studies (1) indicate that alginic acid (2–4), the sulfated polysaccharide fucoidan (5–7), and various phlorotannins, all algal polyphenols (8), are the most abundant constituents of this alga. Some of these algal constituents are known to possess antiviral properties. For example, a multicomponent crude form of fucoidan, available commercially, is reported to act in vitro as a potent inhibitor of HIV (9–12). Polyphenols of higher plant origins also inhibit the cytopathic effect of HIV (13,14) and other higher plant constituents inhibit RT (15). Since, apparently, most of the reported constituents of *F. vesiculosus* are potential candidates for the observed anti-HIV activity, a method to separate and isolate these compounds was developed.

Although several methods for the isolation of fucoidan (5–7), alginates (2), and brown algae polyphenols (8) are reported in the literature, none allows stepwise separation of the potentially active molecules from one single marine algal species. The method presented in this work does permit isolation of these three classes of compounds from a single source, the H_2O-soluble extract of *F. vesiculosus*. Using this method, which relies heavily on differential solubility of the algal components, we initiated large scale testing of the antiviral properties of these groups of compounds.

Also, commercially available fucoidan was fractionated by this method as an external marker for this new process. The separation scheme developed relied on the presence

of a negatively charged sulfate group on the fucoidan molecule. Theoretically, the presence of this charged sulfate should facilitate fractionation by an anion exchanger such as QAE-Sephadex A25 in its basic form. As discussed below, some of these theoretical considerations were not found to be experimentally correct.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Cc was monitored using an LKB Uvicord S Photometer set at 254 and 278 nm, and fractions were collected using an LKB Radi-Rac fraction collector. Hplc was performed on a Shodex Ionpak S-804 analytical column attached to a Waters 601 HPLC pump with an Automated Gradient Controller Model 680 and connected to a Waters Lambda Max Model 481 LC Spectrophotometer set at 207, 257, or 278 nm. Data was evaluated with a Waters Data Module 730. Ir were performed as KBr pellets on a Perkin-Elmer Spectrophotometer Model 28.

EXTRACTION.—*F. vesiculosus* (3.5 kg) was collected in the Bay of Kiel in September 1987 and identified by Ms. E. Kaminsky. A voucher specimen is on deposit at the Phycological Herbarium of the Institute of Marine Science, University of Kiel.

INITIAL EXTRACTION TO OBTAIN EXUDATES.—Fresh plant material was initially washed with tap H_2O at room temperature, and the alga was subsequently frozen at $-20°$ for 20 h. After thawing, the material was kept at 20°, and 10 liters of distilled H_2O was added. After 2 h the H_2O was decanted, filtered, and labeled as the exudate fraction. This fraction was chromatographed on an XAD_2 (3 × 20 cm) column equilibrated with distilled H_2O. The initial fractions were discarded and the column eluted with an H_2O/MeCN linear gradient (0→50%). Eight fractions were collected (F1–8) as illustrated in Scheme 1.

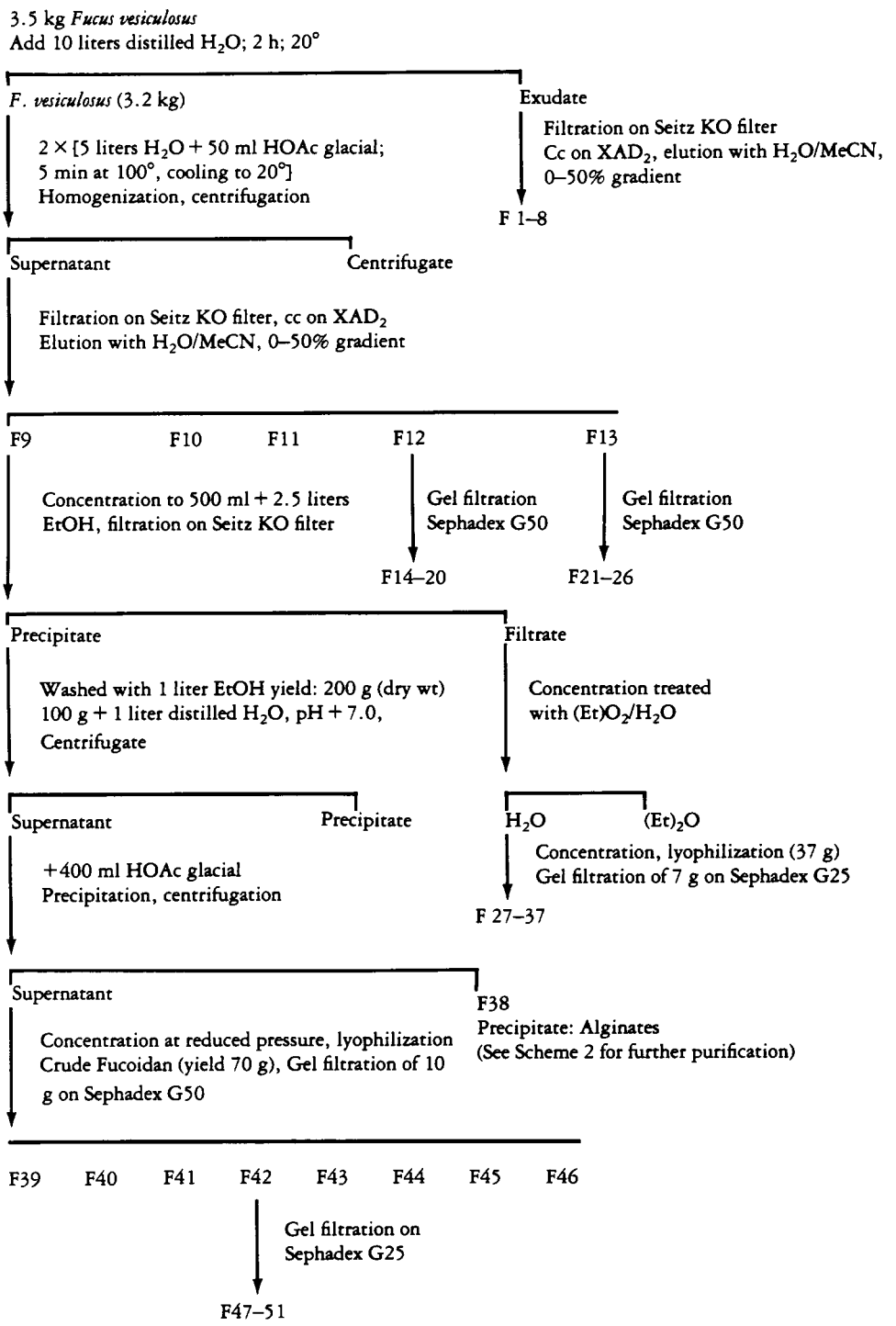
FURTHER PROCESSING OF ALGA.—To the washed alga from above was added 5 liters distilled H_2O containing 50 ml HOAc, and the mixture was boiled from 5 min. The mixture was cooled to 20° and the liquid decanted. The plant material was ground with a meat grinder and homogenized with an additional 5 liters distilled H_2O, and the entire mixture was heated for 1 min at 100°. After cooling to 25°, the homogenate was centrifuged (1400 × *g*) for 10 min in a preparative MSE Centrifuge (Model GF-8). The two supernatants were combined to give a volume of ca. 12 liters and recentrifuged (13000 × *g*) in a Beckman J21 Centrifuge.

ISOLATION OF COLORED "POLYPHENOL" COMPOUNDS.—The entire algal extract was eluted in an XAD_2 (5 × 20 cm) column using an H_2O/MeCN gradient from 0 to 50%. The first fraction (F9) was a "non-adsorbed" fraction, followed by colored compound fractions F10–13. Each of the fractions was tested for anti-HIV activity and each proved active. The most active fractions (F12 and F13) were further individually concentrated and gel-filtered on a Sephadex G50 column (4 × 46 cm) using 0.3 M HOAc as the solvent. The adsorbed compounds were eluted with distilled H_2O and finally with a 1% NH_4OH solution from the Sephadex G50 column. Fractions F14–20 were obtained from F12 and fractions F21–26 from F13.

ISOLATION AND FRACTIONATION OF EtOH-SOLUBLE MATERIAL.—Fraction F9 above was concentrated to 500 ml at reduced pressure, and EtOH (2.5 liters) was added so as to precipitate insoluble material. This was allowed to stand at $-20°$ for 12 h and then filtered on a Seitz K0 filter. The filtrate was concentrated at reduced pressure to 150 ml and partitioned with 200 ml Et_2O. The aqueous layer was treated with 1 g of charcoal, filtered, concentrated, and lyophilized. This fraction proved active in the anti-HIV test, and part of it (7 g) was successfully gel-filtered on a Sephadex G25 column, resulting in fractions F27–37.

ISOLATION OF ALGINATES.—The precipitate from above was washed with 1 liter EtOH and air-dried and yielded 200 g dry wt of precipitate. Some of this (100 g) was dissolved in 1 liter of distilled H_2O, and the pH was adjusted to 7.0 with a dilute NH_3 solution. After centrifugation (13000 × *g*), the nonsoluble material was discarded. To the supernate was slowly added 400 ml of glacial HOAc with stirring whereby a precipitate formed. The precipitation of the alginates was completed after 12 h at 4°. The precipitated compounds were further purified to yield fraction F38.

ISOLATION OF FUCOIDANS.—The supernatant from the glacial HOAc precipitation was concentrated at reduced pressure, freeze-dried, and further purified by gel filtration on a Sephadex G50 column (7 × 110 cm, 0.3 M HOAc, 7 ml/min), whereby eight fractions were obtained (Figure 1A). These were further grouped into high mol wt fractions (F39–41), an intermediate mol wt fraction (F42), and low mol wt fractions (F43–F46). Each high mol wt fraction was chromatographed separately on a QAE Sephadex A25 column (1.5 × 18 cm, 0.01 M NH_4OAc pH 8.0, 1.0 M HOAc, 0.3 M HCl, flow 2 ml/min). The non-adsorbed fractions of each of these were separately chromatographed on a Sephadex G100 (5 × 60 cm)



SCHEME 1. Procedure 1: Preparation of fucoidan, alginates, and polyphenols from *Fucus vesiculosus*.

column. The intermediate mol wt fraction (F42) was chromatographed on a Sephadex G25 column (4 × 40 cm, 0.3 M HOAc, flow 6 ml/min) and resulted in five fractions (F47–51).

FRACTIONATION OF COMMERCIAL FUCOIDAN.—Commercially available fucoidan (Sigma Lot No.

F 5631) was partially purified using the same procedures outlined above. As seen in Figure 1, B and C, this fucoidan preparation was a very inhomogeneous mixture of compounds, including polysaccharides, polyphenols, and low mol wt organic compounds. However, using this method we obtained fractions with chromatographic characteristics similar to those of the fucoidan fractions obtained from the algae (Figure 1A).

FURTHER FRACTIONATION OF ALGINATES.—By taking account of the solubility of alginate molecules in dilute NH_4OH or NaOH at pH 8.0–9.0, further fractionation was performed on fraction F38 (Scheme 2). Ten grams of F38 dissolved in 300 ml of dilute NH_4OH at pH 9.0 was dialyzed against distilled H_2O, and the non-dialyzable material was passed through an XAD_2 column (2 × 20 cm) equilibrated with distilled H_2O. The non-adsorbed main fraction was chromatographed through a QAE-Sephadex A25 column (2 × 20 cm) equilibrated with 0.01 M NH_4OAc (pH 8.0). The non-adsorbed fraction from the QAE-Sephadex A25 column was concentrated to 200 ml and precipitated at room temperature with 20 ml glacial HOAc. The supernatant (F52) was concentrated and gel-filtered on Sephadex G50 (7 × 110 cm) column. Four fractions were collected from this column (F53–56).

MODIFICATION OF ABOVE METHOD TO OBTAIN FUCOIDAN FRACTION.—Alga (14 kg) was processed using a modification of the above procedure (Scheme 1) and produced crude fucoidan from the superficial algal mucus and from the boiled and homogenized algal cell fragments. Details are in Scheme 3.

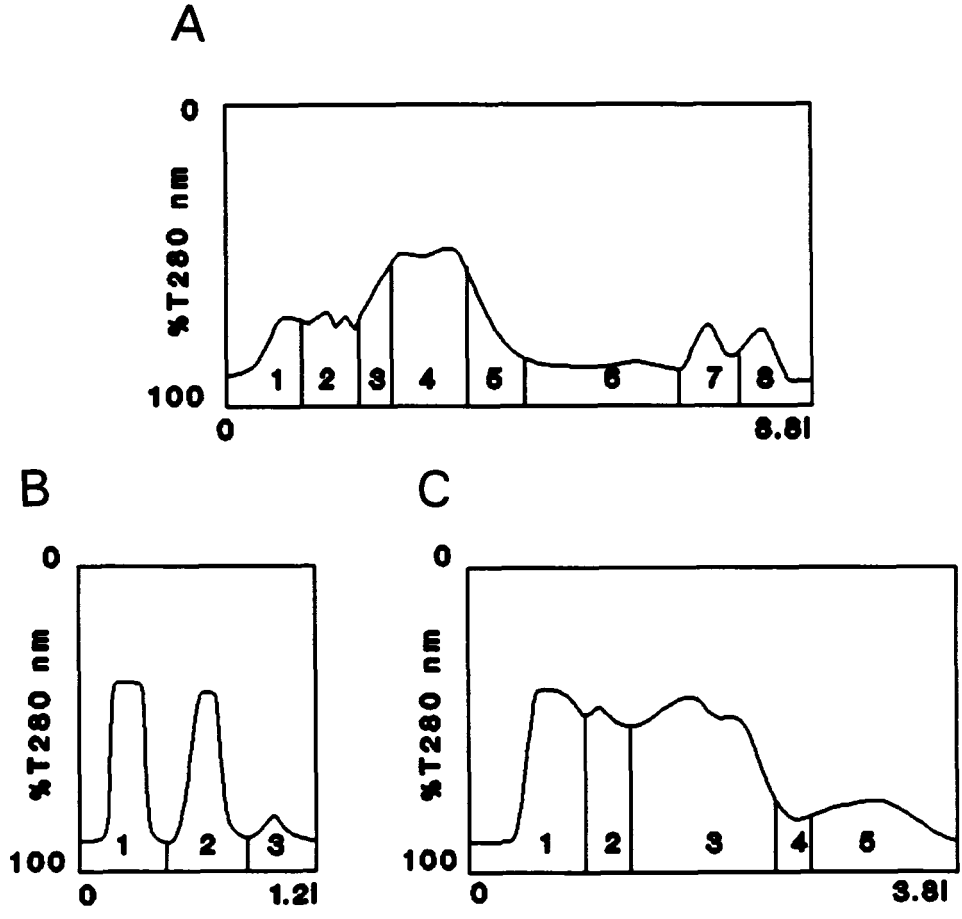
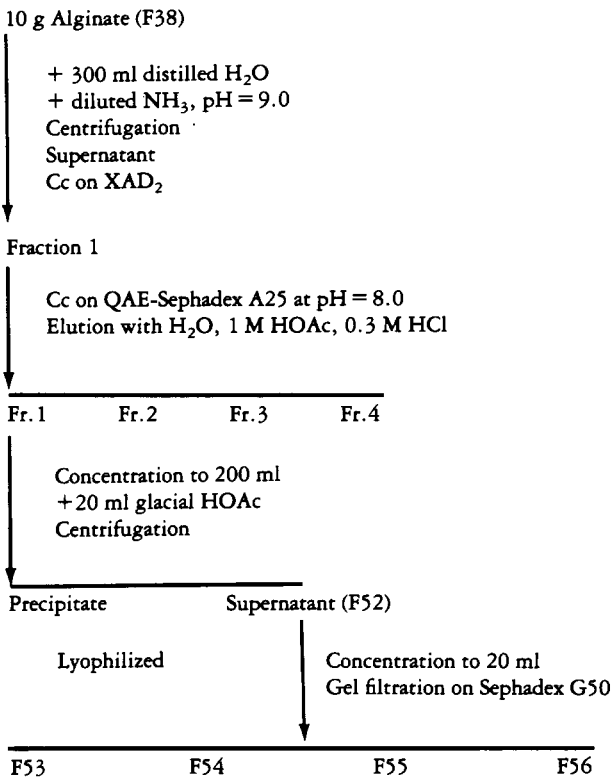


FIGURE 1. Chromatographic comparison of algal "crude fucoidan fraction" to commercial fucoidan (Sigma). A. Gel filtration of "crude fucoidan fraction" from extraction of algae on Sephadex G50 column (7 × 110 cm). Eluting solvent 0.3 M HOAc; flow rate 7 ml/min. B. Chromatography of fucoidan (Sigma) on XAD_2 resin column (2 × 25 cm). Eluting solvent H_2O/MeCN 0→50%; flow rate 5 ml/min. C. Gel filtration of "unabsorbed" Fraction 1 from Figure 1B on Sephadex G50 column (7 × 110 cm). Eluting solvent 0.3 M HOAc; flow rate 7 ml/min.

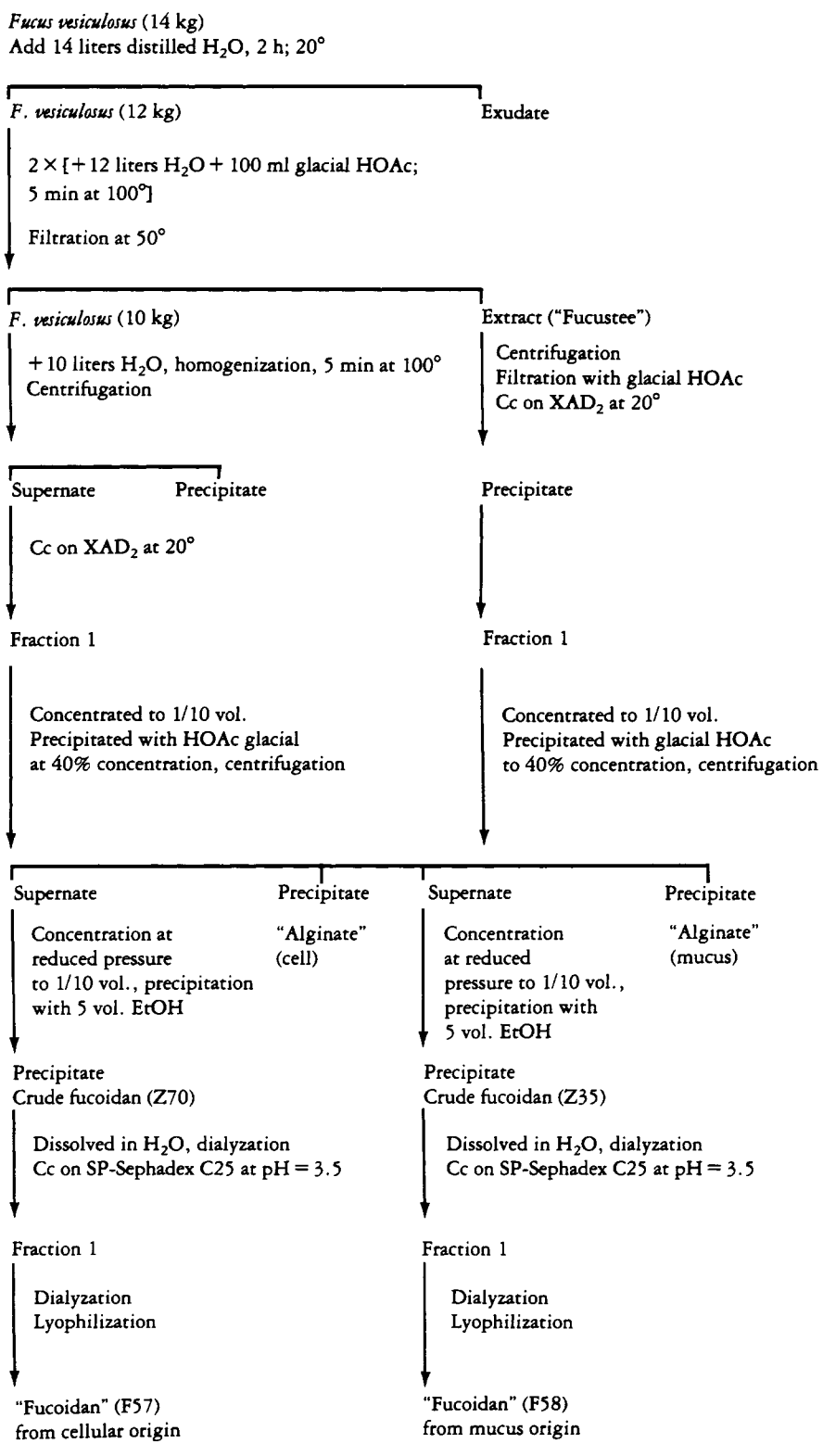


SCHEME 2. Further purification of the alginate fraction F38 of Scheme 1.

TESTING FOR SULFATE.—The appropriate fractions were tested for sulfate qualitatively by a modification of the Lassaigne (16) method using sodium nitrosyl-prussiate.

INHIBITION OF HIV-1 REVERSE TRANSCRIPTASE ENZYME ACTIVITY BY TEST FRACTION.—HIV-1 (LAV-1, strain, obtained from Dr. L. Montagnier, Institute Pasteur, France) was grown in CEM cells (17). Infected cell supernatants (1 ml) were clarified by low speed centrifugation. The virus was pelleted at 145,000 × *g* for 30 min and resuspended in 100 ml of 50 mM Tris (pH 7.8), 0.8 M NaCl, 20% glycerol, and 0.5% Triton X-100. Part of this preparation (20 µl) was incubated in 0.18 M DTT (BRL), 0.006 mg/ml poly A-oligo dT (Pharmacia-LKB), dATP (Pharmacia-LKB), and ^{3}HTTP (20 Ci/m mol) (New England Nuclear), either in the presence or in the absence of test fraction (50 µg/ml). After 3 h of incubation at 37°, the reaction was stopped with cold 10% TCA (Sigma), and the precipitate was filtered and counted (17).

INHIBITION OF HIV-INDUCED SYNCYTIUM FORMATION.—The HIV syncytium-inhibition assay (18) was used to screen individual fractions for anti-HIV activity. Briefly, 96-well microtiter plates (Costar) were coated with 50 µl of a 50 µg/ml poly-L-lysine solution (Sigma). After 1 h of incubation at room temperature, the plates were washed with physiologic buffered saline solution. CEMss cells, as described by Nara and co-workers (18), were plated at a final concentration of 75,000 cells/well in RPMI 1640 medium containing 10% fetal calf serum. Cells were allowed to attach for 30 min at 37° under a 5% CO_2 atmosphere. Media were then removed, and 0.05 ml of HIV-1 [1500 syncytium forming units (SFU) per ml] were incubated in duplicate with the adherent CEMss cells for 60 min at 37°, 5% CO_2. Following this incubation, the virus/medium mixture was gently aspirated and replaced with either fresh medium or medium containing 10 ug/ml of the test fraction. The wells were examined for the presence of SFU after 2 days of incubation at 37°, 5% CO_2. Syncytia were counted using an inverted microscope at 250×. Results were expressed as percent of inhibition of syncytium over control (infected but untreated) cells. Control, uninfected CEMss were incubated either in the presence or in the absence of the test fraction, and were observed for cytopathic effects. Cell toxicity was measured by Trypan-blue exclusion. AZT was used as a positive control for inhibition of syncytium formation. The concentration required to inhibit 50% of the HIV-induced syncytia formation (ED_{50} values) were obtained as described (18).



SCHEME 3. Procedure II: Separate preparation of fucoidan and alginates from the superficial mucus and from the algal cell extract

RESULTS

The exudate extracted from intact algae with cold H_2O (20°) contained predominantly colored plant pigments and polyphenols. It resolved chromatographically into eight fractions (F1–8). With the exception of F5 and F8, all inhibited HIV reverse transcriptase and HIV-induced syncytium formation (Table 1). However, these active fractions were also cytotoxic and were not further investigated. All other fractions listed in Table 1 were not cytotoxic at the concentration used in the syncytium inhibition assay.

Hot H_2O treatment of the alga disrupted the rigid polysaccharide cell wall and rendered it easily homogenizable, producing a red-brown crude algal extract containing, together with all polysaccharides and proteins, the soluble polyphenols and plant pigments.

The majority of the colored compounds adsorbed to the polystyrene XAD_2 resin separated from the polysaccharides and the polar compounds. The latter readily passed

TABLE 1. Percent Inhibition of HIV-1 Reverse Transcriptase (RT) and Syncytium Formation by Test Fractions.[a]

Test Fraction	% RT inhibition	% Inhibition of syncytium
F1	89	95
F2	94	97
F3	92	30
F4	95	82
F5	88	0
F6	92	56
F7	90	95
F8	92	0
F10	89	33
F16	87	56
F17	90	90
F18	81	0
F19	90	0
F25	94	54
F26	92	82
F32	93	0
F33	94	31
F34	96	51
F35	90	0
F36	86	33
F37	90	8
F38	85	66
F39	91	31
F40	93	92
F41	84	51
F43	89	95
F44	92	85
F47	63	45
F49	95	4
F57	15	80
F58	35	98
AZT (control)	N.D.	100

[a]All compounds were tested for inhibition of RT and of syncytium formation at the non-cytotoxic concentrations of 50 μg/ml and 10 μg/ml, respectively. RT counts of positive control = 27,800 cpm. Infected control contained 75 to 100 HIV-1 syncytium-forming units per well. N.D.: not done.

through the XAD_2 column. The H_2O-soluble, inhomogeneous, red-brown colored substances, including the polyphenols, were separated by gradient elution with MeCN and thereafter by gel filtration on Sephadex G50. Several anti-HIV active fractions were eluted from the Sephadex G50 column first with 0.3 M HOAc, then with distilled H_2O, and finally with 1.0% NH_4OH solution. These fractions inhibited HIV-RT and syncytium formation at concentrations that were not cytotoxic. Further evidence that these fractions were polyphenols was obtained using ir spectroscopy. The ir spectra obtained from the colored polyphenols were all similar to each other and similar to the ir spectra for the phlorotannins from *F. vesiculosus* obtained by Ragan (19). Two of these colored compounds were highly active (F17 and F26).

Concentration at reduced pressure followed by precipitation with EtOH separated the polysaccharides from the low mol wt, EtOH-soluble, material. Gel filtration on Sephadex G25 of this supernatant produced several fractions, only one of which (F34) was active against HIV.

The HOAc precipitate (F38), considered to contain the nonsulfated alginates, also showed anti-HIV activity. Solubility at pH 8 allowed further chromatographic purification of this fraction (Scheme 2). The major anti-HIV active component of fraction F38 did not bind to XAD_2 or to QAE-Sephadex A25 columns (both at pH 8), indicating a polar but neutral (or cationic) property. As seen in Scheme 2, a subfraction of F38 (F52) was gel-filtered on Sephadex G50, yielding four fractions (F53–56) all of which were active against HIV. These fractions were free of sulfur.

After precipitation with glacial HOAc, the relatively non-viscous crude fucoidan was in the supernatant. Purification of this crude fucoidan component by gel filtration on Sephadex G50 produced eight fractions (F39–46). Of these, the ones with higher mol wt (F39–41) exhibited anti-HIV activity and were individually further chromatographed on QAE-Sephadex A25 at pH 8. Unexpectedly, none of these fractions active against HIV would bind to the QAE-Sephadex A25 column, showing neutral polysaccharide characteristics. The material retained on the QAE-Sephadex A25 column (eluted with 1 M HOAc and with 0.3 M HCl) was inactive in the two anti-HIV tests. Similarly, none of these active fractions was retained by SP-Sephadex C25 column. They were further purified by gel filtration on Sephadex G100 and lyophilized. These active fractions appeared to have a high degree of purity, as revealed by the presence of only one main peak in the hplc chromatograms obtained using an analytical Shodex Ionpak S-804 column (data not shown). All the fractions obtained from the above procedures contained sulfur. The ir spectra obtained from the colorless sulfated polysaccharides revealed similarities to the ir spectra of brown algal polysaccharides published elsewhere (20).

The intermediate mol wt fraction (F42) was also active against HIV, and two of the fractions (F47 and F49) obtained from a Sephadex G25 separation of F42 inhibited RT. All fractions from this column contained sulfur.

The modification of procedure 1 described above (see Methods) produced crude fucoidan and alginate fractions from the superficial algal mucus and from the boiled and homogenized algal cell fragments. Two main anti-HIV activie fucoidan fractions were obtained: F57 from algal cell fragments and F58 from algal superficial mucus (Scheme 3). Both fractions inhibited HIV syncytium formation at noncytotoxic concentrations and were shown to contain sulfur. The ED_{50}'s obtained for F58 and F59 were 1 μg/ml and 2.5 μg/ml, respectively.

Fractions from commercial fucoidan had anti-HIV activity and did not bind either to XAD_2 or to QAE-Sephadex A25, indicating again a neutral charge for these components. Further purification of these fractions on Sephadex G100 showed a high degree of purity as indicated by hplc analysis (data not shown).

DISCUSSION

The present work was undertaken to develop a new and more productive method for the isolation of the polysaccharides, polyphenols, and alginates from the marine brown alga, *F. vesiculosus*, for large scale anti-HIV testing. As indicated in the results section, this was achieved, and in contrast to earlier procedures, chromatographic separations and fractionation of these fractions produced sufficient quantities of different biologically active underivatized, natural compounds. Of particular interest was the discovery of anti-HIV activity in multiple chemical fractional groups.

As shown in Table 1, some fractions inhibited the HIV-RT enzyme but did not inhibit HIV syncytium formation. It is likely that the active compounds were not able to enter the infected cell. At the screening concentrations tested, the majority of the active fractions inhibited both RT enzyme activity and syncytium formation. However, experimental data obtained in our laboratory (E. Kraiselburd *et al.*, manuscript in preparation) showed that syncytium formation was inhibited at lower concentrations than those required for HIV-RT inhibition. ED_{50} values of 1.0 and 2.5 ug/ml were obtained for F57 and F58, respectively. However, a very modest inhibition of HIV-RT activity was obtained by these fractions at the concentration of 50 μg/ml (Table 1). Therefore, some of these fractions may have inhibited syncytium formaton by mechanisms that did not involve the virus RT enzyme.

During fractionation, two unexpected observations were encountered. The first was that the active fractions of the sulfated polysaccharide fucoidan did not bind on the anion exchanger QAE-Sephadex A25 at pH 8. Also, the alginate fraction behaved in the same way. Because of the strong negative charge present in the alginate molecules and sulfated polysaccharides at pH 8, the electrostatically inert behavior of these two anionic polymers may arise from a steric hindrance between the large anionic polysaccharide molecules and the polysaccharide matrix of the anion exchanger which counteracts interionic binding. Alternatively, the investigated polysaccharide may exhibit neutral (or basic) properties. However, a basic character for the fucoidan could be easily excluded by its chromatographic behavior on a cation exchanger column (SP-Sephadex C25). At pH 3.5 it passed unadsorbed through this column. The alginic acid molecules are nearly insoluble at pH 3.5 in H_2O; therefore they could not be chromatographed on SP-Sephadex C25.

The most active anti-HIV fucoidan fraction from both our algal extracts and the commercial source was repeatedly present in the second void volume of a gel filtration scheme on Sephadex G50. Our calculations indicate that this active anti-HIV polysaccharide has an intermediate mol wt of 10,000–20,000 daltons.

A possible explanation for a neutral character of the sulfated polysaccharide fucoidan could be an additional esterification of the $R\text{-}O\text{-}SO_3^-$ groups. This may happen intramolecularly with free OH groups of the polysaccharide, or also intermolecularly with the precipitating EtOH during the fractionation.

Our second unexpected observation was the anti-HIV activity of a few sulfate-free polysaccharide fractions (F52–56) derived from the alginate precipitate F38 (Scheme 2). This was the first indication that a sulfate-free polysaccharide had antiviral activity. Future analysis of these fractions may contribute to a better understanding of the mode of action of the polysaccharides as a group. Indeed, the anti-HIV activity of neutral polysaccharides has been disputed and denied in the literature (21,22). An understanding and interpretation (23) of the biological activity of sulfated polysaccharides from marine algal resources has already extended our knowledge on the mode of action of natural and synthetic sulfated polysaccharides (24–37, 21), and we expect it will continue to do so. The main point raised by these and other investigations is the role that the

strongly negatively charged R-O-SO_3^- groups have on the specific binding of sulfated polysaccharides to the basic V3 loop of the gp120 molecule, which inhibits HIV attachment to the CD4 receptor. Alternatively, sulfated polysaccharides may inhibit the events subsequent to virus attachment to the CD4 receptor (38). Only the compound HOE/BAY 946, a polysulfated polyxylan, may differ in its mode of action from the other sulfated polysaccharides. It acts pleiotropically by increasing the hydrophobicity of the lymphocyte membranes suppressing HIV-protein synthesis and reducing release of HIV particles from the infected peripheral blood mononuclear cells in vitro (39,40). Until further structural and mechanistic studies are performed, we have no explanation for the anti-HIV and for the well established anticoagulant effect of the fucoidan subfractions isolated (41).

We should emphasize that the commercially available fucoidan (Sigma Lot No. F5631) behaves chromatographically in the same way as our fucoidan preparation. The major anti-HIV activity was in this case concentrated within a deionized fraction of intermediate mol wt and eluted also in the second void volume.

The purification of the biologically active algal polysaccharides was complicated by their electrostatically inert but otherwise strong polar character. Neither anion exchange chromatography (with gradient elution) nor hplc on reversed-phase material could be used for their purification. However, we solved this purification problem by subsequent gel filtration on Sephadex G50 and Sephadex G100, and finally by hplc on a Shodex Ioanpak S-804 column, which also behaves as a molecular sieve.

Although the chemical structure of fucoidan was published in the early 1950s (42–44), it is worthwhile to mention that these previous studies were always carried out after hydrolysis of crude fucoidan preparations. Up to now, no polysaccharide molecules from *F. vesiculosus* have been isolated as pure compounds for structural or chemical studies. Actually, fucoidan is presently considered to be a complex mixture, as suggested by Figures 1B and 1C for the commercially obtained fucoidan.

In summary, the procedures described here revealed that the brown alga *F. vesiculosus* is a rich source of anti-HIV active polysaccharides and polyphenols, whose partial purification has been finally elaborated. We believe the methods described for their purification may be used for the isolation of biologically active polysaccharides and polyphenols from other algal species and perhaps other plants.

ACKNOWLEDGMENTS

This work was supported by NIH/MBRS Grant No S060RR08224 and Sea Grant RIMB-12-10. The support of the Verband der Chemischen Industrie (Germany) is highly appreciated. Part of his work is in partial fulfillment of the Ph.D. Thesis of Anne Béress. We thank Mr. Juan Oquendo and Mr. Horacio Serrano for their excellent technical assistance and Ms. Olga Cortez for typing this manuscript.

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Received 13 July 1992