PLANT VIRUS INHIBITORS FROM MARINE ALGAE

by

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ABSTRACT

This study was the first to evaluate new world algal species for compounds with potential for plant virus chemotherapy. Methanolic extracts from 30 species of marine algae were assayed for antiviral activity against potato virus X (PVX) in local lesion assays using Chenopodium quinoa L. as host. Extracts from six algal species (Fucus gardneri Silva, Alaria nana Schrader, Ralfsia sp. (Berkley), Codium fragile (Suringar) Hariot, Fragilaria oceanica Cleve, Egregia menziesii (Turner) J.E. Areschoug) inhibited PVX infectivity by more than 80%, with a disproportionate number of these extracts coming from the phylum Heterokontophyta. Of the six most active extracts, it is the first time that Fucus gardneri, Ralfsia sp. and Fragilaria oceanica have been reported as sources of antiviral agents. Antiviral activity from the most potent extract, F. gardneri, was selected for phytochemical analysis. Fractionation of the crude extract resulted in the isolation and identification of the polysaccharide alginate as a source of bioactivity. Alginate inhibited potato virus X infectivity by 95%, and preliminary transmission electron microscopy indicates that the mode of action may be related to aggregation of virus particles. Bioactivity in a second fraction was the result of phenolic and amino-based compounds that have yet to be positively identified.

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This thesis is dedicated to my Family

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Chapter⁽¹⁾

Introduction to the Field

All that we do is touched with ocean, yet we remain on the shore of what we know...

-Richard Wilbur, former U.S. poet laureate

Background

As agriculture has developed into an intensive and global industry, plant virus outbreaks and chronic infections have become increasingly frequent and disruptive (Thresh, 1983). Economic and social costs resulting from such crop pathogens call for better means of either virus prevention or control (Bos, 1999). Traditionally the agricultural industry has looked toward synthetic chemistry to solve problems of crop disease and fertility. However, natural chemical diversity is playing an increasing and integral role in the search for novel and alternative agrochemicals (Wedge and Camper, 2000). Plant viral chemotherapy is currently an effective strategy used to fight plant virus infection, however problems with phytotoxicity limit its application (Hansen, 1989). Exploiting natural chemical diversity to meet the demand for alternative, non-toxic plant antivirals presents an excellent opportunity for discovery of new products (Muller *et al.*, 2000). The goal of this project is to evaluate the chemical diversity of selected British Columbian marine algae for antiviral activity against a plant virus, potato virus X.

AGRICULTURAL SIGNIFICANCE

For many plant virologists, it is the agricultural significance of viruses that ultimately drives most lines of investigation. Plant viruses are very important crop pathogens, second in importance only to fungi, and result in annual losses in the billions of dollars (Matthews, 1991). The effects of infection are manifested in a variety of ways. The most obvious is often a reduction in growth that is frequently accompanied by lower crop productivity. Plant virus infection can also affect crop quality. Changes in chemical composition such as sweetness or taste, color, and size can all lead to losses for the grower. As is the case with some human viral infections, plant viruses may also increase host vulnerability to stress. By reducing vigor, viruses can leave crops vulnerable to opportunistic infections, competition, drought or frost. An example of the scale of potential social and economic consequences is provided by the cacao swollen shoot badnavirus in West Africa. Between the discovery of the disease in the 1930's and its control in 1977, Ghana alone lost over 160 million cacao trees (Bos, 1999). More recently, pepino mosaic virus has emerged as a new crop pathogen for tomato growers in both Europe and North America. Although economic impacts to date have been negligible, the threat of the virus jumping into more important solanaceous crops like potato is significant (EPPO, 2001). Given the impact viruses have on the economics and trade of agricultural products there is clearly a need for practical alternatives to prevent or curtail infections.

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PLANT VIRUSES

Viruses are difficult to define but are unique in almost all respects. Not only are they very small in size and simple but their means of replication, translocation and transmission set them apart from all other pathogens. Their ability to cause disease, carry genetic material and reproduce suggests behavior characteristic of other microorganisms. However, in other respects viruses resemble complex chemical molecules in that they are essentially lifeless outside host organisms. In their most basic form viruses are composed of nucleic acid, either RNA or DNA, surrounded by a protective capsule of protein and, in some groups, an additional membrane. The nucleic acid may exist in either single or double stranded configuration, although for plant viruses the most common form of genome is positive single-stranded RNA (Matthews, 1991). The simplicity of viruses however imparts limitations to their natural history. For viruses to perform any metabolic function they must associate with the cellular machinery of another organism, and it is in the process of doing so that viruses can cause disease. The symptoms that are recognized as a disease are often the result of virus particles disrupting host cellular function, consuming cellular resources and occupying cellular space (Agrios, 1997).

DISCOVERY OF VIRUSES

Viruses and other cellular parasites such as viroids are the most recent class of plant pathogens to be discovered (Hansen, 1989). One of the earliest well documented reports of viral disease in agriculture dates back to seventeenth century Holland. Although at the time not known to be caused by a virus, tulips infected with the tulip breaking potyvirus were highly prized for their colorful and streaked petals (Walkey, 1991). The true start of plant virology began in the late 19th century with the identification and characterization of the first virus, tobacco mosaic virus (TMV), by Beijerinck. (1898). This new class of transmissible agents became known collectively as filterable viruses, because they could easily be passed through ceramic filters designed to remove bacteria (Bos, 1999). Over time the definition of a virus has become refined to differentiate viruses from other cellular parasites, such as Rickettsiae, Chlamydiae and Mycoplasmas. Matthews (1991) defines viruses as:

"A virus is a set of one or more nucleic acid template molecules, normally encased in a protective coat or coats of protein or lipoprotein, that is able to organize its own replication only within suitable host cells. Within such cells, virus replication is (i) dependent on the host's protein-synthesizing machinery,

(ii) organized from pools of the required material rather than by binary
fission, (iii) located at sites that are not separated from the host cell contents
by a lipoprotein bilayer membrane, and (iv) continually giving rise to variants
through various kinds of change in the viral nucleic acid."

VIRAL TRANSMISSION

Inactive outside of their host organisms, plant viruses must rely on outside mechanisms for dispersal and transmission. The discovery, in 1883, that

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leafhopper insects could transmit rice dwarf disease was the first time that viral transmission was demonstrated (Zaitlin and Palukaitis, 2000). Since then, plant viruses have been found to be transmissible by many different means. Of the 997 plant viruses known, many can be transmitted to successive generations through seed, pollen and vegetative propagation; this is known as vertical transmission (Bos, 1999). Intermediate organisms can also facilitate viral transmission; fungi, bacteria, invertebrates and vertebrates including humans often serve as vectors for plant viruses; this is known as horizontal transmission (Matthews, 1992). The unintentional spread of viruses through human activities such as grafting, vegetative propagation, mechanical damage and global trade are often important contributors to plant virus epidemics. In fact, epidemiological studies have found human activity is a leading cause of plant virus outbreaks (Thresh, 1983). An example of such is the outbreak of plum pox potyvirus in the Niagara Peninsula, Ontario in 2000 (CFIA, 2001). It is thought that infected rootstock was imported into Canada and once in the country, aphids may have transmitted the virus to adjacent orchards. Ultimately all infected orchards may have to be destroyed and replanted with virus-free trees or alternative crops (Cree, 1999). Such outbreaks are not infrequent and their occurrence demonstrates the value of surveillance of imports and early detection in the field.

DETECTION & DIAGNOSIS

The development of improved methods for the detection and characterization of plant viruses has been of immeasurable importance to this

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field. Prior to the development of serological and molecular techniques, determination of suspected viral agents relied on using indicator hosts, testing transmissibility to indicator species, host range, cross protection tests and description of cytological effects (Matthews, 1991). The development of the local lesion assay by Holmes in 1929 permitted for the first time the determination of virus infectivity. Although this technique is limited by sensitivity, it is still the only method that can discriminate between viable and nonviable plant virus particles (Zaitlin and Palukaitis, 2000). The development of serology and reverse transcriptase polymerase chain reaction (RT-PCR), has led to applications such as immuno-labeling and molecular screening using virus-specific primers. These methods have considerably advanced characterization in plant virology (Walkey, 1991; James *et al.*, 1997). For the purposes of this study, diagnostic needs were met using local lesion assays and enzyme linked immunosorbent assays (ELISA).

CONTROL MEASURES

Despite the importance of viruses in crop pathology there are few methods available to prevent infection. To compound the situation for growers, there are no practical field methods for treating virally infected crops (Bos, 1999). The most common and successful approach to virus control is prevention. On a global scale, regulatory measures such as quarantine and inspection help to limit the spread of viruses (Agrios, 1997). In the field, preventive measures include crop rotation, greenhouse and field hygiene, limiting mechanical damage and the removal of symptomatic plants and related taxa that may serve as virus reservoirs. Biological control of vectors, planting of barrier crops to intercept vectors and planting with certified virus-free seed are other passive measures available to growers (Matthews, 1991, 1992). The last method raises a key point, for passive controls to be effective the crop must first be free of infection.

PRODUCTION OF VIRUS-FREE PLANTS

For vegetatively propagated crops, such as potato or fruit trees, viral infections are often a persistent problem because viruses may spread to successive generations through the propagules themselves. However, once the cycle is broken, crops can often be maintained virus-free for several growing seasons. Obtaining virus-free foundation stock from which propagules are generated can be achieved using a number of approaches (Matthews, 1992).

Traditional Methods

Early methods took advantage of individual plants that were naturally virus-free because of either geographic isolation or chance. Although seemingly inadequate, this method successfully provided for early potato and fruit tree cultivation (Hansen, 1985). Since 1950, heat therapy has been used successfully to rid plant material of viruses. Using this method, dormant or growing plants are exposed to above average temperatures (usually 35-54 °C) for a period of weeks (Matthews, 1991). Viral suppression achieved using heat therapy can be attributed to the disruption of viral multiplication. At temperatures above optimum, viral

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multiplication is reduced while viral degradation and plant growth continue. This can lead to the production of virus-free tissue that can be used to establish foundation stock. However, low plant survival, host intolerance, viral resistance and length of the process limit the practical application of heat therapy (Hansen, 1985).

An alternative approach to obtaining virus-free tissue is meristem tip culture (Walkey, 1991). This technique takes advantage of a natural phenomenon which for some viruses leaves the apical 100-500 μ m of many shoots virus-free (Matthews, 1991). It has been hypothesized that this phenomenon is the result of either cell division exceeding the rate of viral synthesis or cell division outcompeting viruses for resources. Despite its value in eradicating certain viruses, recalcitrant infections and practical limitations such as manipulation of the meristem and poor regeneration make meristem culture best suited for application in combination with other techniques (Hansen, 1989).

Breeding Resistance

Breeding crops with resistance to plant viruses provides a completely different approach to this problem by preventing rather than treating infection. Originally this was achieved by introducing genes for resistance, tolerance or hypersensitive response into cultivars through crossbreeding. The hypersensitive response can prevent systemic infection by confining the proliferation of viruses to the site of entry. Unfortunately because of breeding limitations, expression of these traits into agriculturally important crops generally failed (Walkey, 1991).

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However when successful, this strategy has proven to be sound. As an example, the breeding of virus resistant sugarbeet cultivars in response to emerging viruses has repeatedly prevented the collapse of the sugar industry in North America. With the introduction of molecular techniques over the past decade, development of virus resistance cultivars is no longer restricted by reproductive compatibility (Bos, 1999).

Genetically Engineered Resistance

Electroporation, gene-gun and other molecular techniques now allow free shuffling of genes not only between species but also between kingdoms. By overcoming limitations of generation time and breeding incompatibility, molecular advances have revolutionized the process of developing resistant cultivars. The most common approach for achieving plant virus resistance in this field is a technique called coat-protein-mediated virus resistance (Bos, 1999). DNA complementary to viral coat-protein genes is introduced and expressed in crop plants. The concept comes from a process called cross-protection. Crossprotection refers to resistance acquired by a plant following a mild viral infection, much like vaccination. By mechanisms not fully understood, once a plant is infected subsequent exposure to the same or a related virus does not result in disease (Miller and Hemenway, 1998).

Genetic engineering techniques have opened up many possible strategies for reducing crop pathogenesis. Examples specific to potato viruses include resistance acquired through transformation with a natural antiviral protein gene from pokeweed and a protease gene from potato virus Y. Both of these genes result in the expression of enzymes that confer antiviral protection to the transformed potato (Kaniewski and Lodge, 1992; Jilka and Tumer, 1996). However, issues over public and environmental safety of genetically modified organisms have raised concerns over the use of transgenic plants in agriculture. Other factors slowing the application of this approach include the large investment required to develop resistant cultivars. Unlike other methodologies, most developments in this field are by their very nature specific to each host-virus combination making cost recovery more difficult (Matthews, 1992). Generally speaking, ethical and proprietary issues as well as concerns over long-term effectiveness have made genetically engineered solutions less desirable at present.

PLANT VIRUS CHEMOTHERAPY

Intervention against viral infection can take three forms: avoidance, prophylaxis, and post-infection treatment (Rozhon *et al.*, 1994). To this point all major antiviral strategies have been reviewed with one important exception, viral chemotherapy. Plant virus chemotherapy offers a further approach to providing virus-free plants for agriculture. Chemotherapy does not have the advantage of genetic resistance that genetic engineering offers, however it also does not carry the disadvantages. Over the past 50 years, plant virus chemotherapy has grown from a string of serendipitous observations into a field with substantial outcomes and potential for further improvement. Promising directions for plant viral chemotherapy include the identification of new effective but non-phytotoxic antivirals and plant defense stimulators (Walkey, 1991; Klarzynski *et al.*, 2000). Currently, plant viral chemotherapy is not practical for field applications. Much like meristem culture and heat therapy, chemotherapy is generally performed under laboratory conditions to the tissues of desirable but infected cultivars (Walkey, 1991). It acts by slowing virus multiplication such that new tissues grown while under therapy are virus-free and can be used to establish uninfected foundation stock (Hansen, 1989).

In contrast to the number of antibiotics available for treating bacterial infections, there are few comparable treatments for viral infections. The intimate association between virus replication and host metabolism poses a problem for the development of viral chemotherapy in both plant and animal systems. Unlike many pathogens, viruses have an internal, obligate relationship with their hosts, depending on host cellular processes for all activity. Consequently, drugs that are intended to inhibit viral function often also act against cellular processes resulting in toxicity to the host (Rozhon *et al.*, 1994).

Difficulty in identifying specific viral targets for drug action and an incomplete understanding of infection and replication processes, at one point made the development of successful viral chemotherapy unlikely (Neushul, 1990). However, in 1961 Quak successful demonstrated in a series of thiouracil treatments that chemotherapy could not only prevent virus replication but could do so without severely affecting cell metabolism. Her strategy was to minimize

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phytotoxicity by using viral inhibitors specific to RNA metabolism (Quak, 1961). To date the most successful plant viral chemotherapeutic compound is a synthetic uracil analogue specific to RNA metabolism, called ribavirin. This compound, also known by the name Virazole, is effective against many viruses including those of animals (Hansen, 1988). However because some cellular disruption still occurs, inhibition by ribavirin is often accompanied by phytotoxic effects including: stunted growth, irregular foliage and limited root development (Bos, 1999). Similarly, DHT (2,4-dioxo-hexhydro-1,3,5-triazine) can reduce losses incurred due to viral infection, however it appears to be unable to eradicate the virus entirely (Hansen, 1988). Resolution of these problems may be found through combination therapy as is increasingly being done with human viruses. By combining two or more therapeutics, the individual concentration of each compound can be reduced and their combined modes of action act together to further challenge the infection (Hansen, 1988).

Resolving the demand for alternative non-toxic antivirals remains a high priority in applied virology. Both pharmaceutical and agrochemical discovery processes are largely geared toward synthetic compounds. This in part is an artifact of the industry's heavy reliance on high throughput screening (HTS) to identify new leads (Muller *et al.*, 2000). This is because HTS is well suited to simple extracts like those produced by combinatorial chemistry, but is essentially incompatible with the complexity of natural extracts. Furthermore, difficulties inherent with purification and structural elucidation also contribute to dominance of synthetic

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therapeuticals; however, advances in automation are making natural chemical diversity more accessible (Grabley and Thiericke, 1999).

Although it is difficult to argue the success of the HTS, this method has disadvantages, an important one being the nature of the substance pool. Evidence suggests that from a strategic point of view, drug and agrochemical research require greater focus on evaluation of natural products. By focusing screening programs strictly on compounds of synthetic origin, particularly from combinatorial chemistry, substantial forms of chemical diversity may be overlooked. Analysis shows that approximately 40% of chemical diversity found in natural products is not available through traditional synthetic approaches (Muller *et al.*, 2000). Furthermore synthesis of highly complex molecules, such as taxol, is simply impractical for screening programs. Consequently, screening programs based on natural products are essential. Natural product screening programs extend the range of available chemical diversity, thus complementing existing programs focused on compounds of synthetic origin.

NATURAL PRODUCTS AND THEIR USES

Once considered to be incidental by-products of metabolism, secondary metabolites are now recognized as mediators of herbivory, competition and a host of other ecological interactions. A sort of chemical warfare appears to have evolved between groups of organisms. As one group would produce a noxious chemical the other would develop detoxifying mechanisms or tolerance. In some cases this has resulted in plants having suites of redundant defensive compounds. Similar strategies are found in other organisms, particularly invertebrates such as insects or sessile organisms such as sponges (Wedge and Camper, 2000). Natural products that are identified as having valuable bioactivity for human use do not necessarily have a corresponding role in the organism from which they come. Rather societies have simply found an alternative use for naturally occurring chemical diversity (Hansen, 1989)

Chemical diversity, generated by such things as defense mechanisms, has been exploited by humans for millennia. India and China are two well-known examples of cultures that relied heavily on plant-based medicine; however, it can probably be said that until recently this was the case for all cultures (Grabley and Thiericke, 1999). Some well-known plant-based medicines are morphine from *Papaver somniferum* and quinine from the Peruvian *Cinchona officinalis* tree. Isolation of such active principles during the early part of the 1800's led to the development of the modern day pharmaceutical industry. More recent examples of natural product based drugs are the anticancer agent, taxol, and the immunosuppressant, FK 506 (Grabley and Thiericke, 1999). The first record of a natural product used to combat plant virus infection dates back to 1925. Duggar and Armstrong found several plant extracts, which curtailed TMV infections in tobacco. Natural products remain a source of bioactive compounds for agriculture. Examples of recent agrochemicals to come out of natural product research include: insecticidal avermectins from *Streptomyces avermitilis* and a new class of non-phytotoxic agro-fungicides based on the fungal metabolites strobilurin and oudemansin (Anke and Steglich, 1999).

NATURAL PRODUCTS AS VIRAL INHIBITORS

Demand for novel, biologically active structures is encouraging chemists to exploit natural chemical diversity (Wedge and Camper, 2000). Although not inherently better than synthetically derived compounds, natural products as mentioned can augment screening programs by providing chemical diversity and variation not easily accessible to synthetic chemistry. The trend towards including natural products in screening programs is becoming apparent in the pharmaceutical market where "60% of approved drugs and pre-new drug application candidates were of natural origin for the period 1989 to 1995" (Grabley and Thiericke, 1999). Furthermore, in 1996 drugs based on natural products accounted for \$79 billion of the \$227 billion market in new pharmaceuticals (Muller et al., 2000). To date only a handful of natural productbased antivirals, such as Neem (Azadiracta indica) and Boerhaavia diffusa, have been put into practical agricultural applications (Hansen, 1989). Considering the chemical diversity only available through natural products and the minor extent to which such diversity has been exploited, future work in this area is bound to yield discovery.

GOALS AND OBJECTIVES

A combination of research capability and proximity to the Pacific coast makes screening organisms from the marine environment for antiviral activity a natural partnership. There were two main objectives of this research project. The first objective was to screen 30 selected marine algae for antiviral agents against the plant virus potato virus X. The second objective was to select from the most antiviral extracts a candidate for more detailed characterization. *Fucus gardneri*, the most potent marine algal extract, was selected. Detailed study of this extract included evaluation for bioactivity against local and systemic PVX infection and phytochemical analysis.

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Chapter 2

Screening Marine Algae for Antiviral Activity

"The answers we are searching for on earth are to be found in the sea" -Anonymous

Introduction

SUMMARY AND OBJECTIVES

Marine algae have received little attention as a source of chemical diversity in the search for non-toxic alternatives to current plant virus chemotherapeuticals. Marine ecosystems offer a largely untapped source of biological and chemical diversity, which over the past thirty years has yielded novel and unique bioactive compounds (Carté, 1996). Several workers have found that marine algae contain compounds with significant potential against human (Nakashima *et al.*, 1987; Beress *et al.*, 1993; Hudson *et al.*, 1999a,b) and plant viruses (Galal *et al.*, 1999; Padmakumar and Ayyakkannu, 1997). Although marine algae have been recognized as a source of bioactive compounds for several decades, it was not until 1997 that the rich flora in the coastal waters of British Columbia was screened for therapeutic value (Kim *et al.*, 1997).

The goal of this study was to identify marine algae that contain non-toxic, antiviral agents for potential use in plant chemotherapy. Work on this project began after algal extracts in a small screening proved to have potent antiviral activity against potato virus X. The study includes more than 30 species of marine algae, mostly macro-algae collected from the shores of Vancouver Island.

MARINE NATURAL PRODUCTS

Appreciation for the potential of marine natural products as therapeutics began in the early part of the 1970s. Marine organisms continue to yield molecules of unique and unconventional chemical structure (McConnell *et al.*, 1994). The reason that marine ecosystems are so richly endowed with chemical diversity is not fully understood, although the answer may lie in the role that oceans played during early evolution (Volkman, 1999). Marine environments gave rise to life on the planet and are home to many "primitive" organisms. It has been hypothesized that because these organisms often lack sophisticated immune systems, survival in such competitive environments requires diverse and often toxic defense strategies (Grabley and Thiericke, 1999).

The rationale behind the suggestion that these secondary metabolites play a defensive role is two-fold. First, synthesis of these structurally complex molecules is metabolically expensive and correspondingly it can be argued must be of some utility to the organism. Second, there is a strong correlation between these unique secondary metabolites and their occurrence in seemingly vulnerable soft-bodied organisms like algae and sponges (Pawlik, 1993). Such defense strategies are often based on metabolites that have very specific binding. This specificity is attractive to agrochemical and pharmaceutical industries, as they try to avoid nonspecific chemical interactions that lead to toxicity and are a chief cause of compounds failing to reach the market (McConnell *et al.*, 1994).

Despite the promising outlook of marine-based therapeutics, the field is still in its infancy, with as yet no commercial products. Two interrelated issues are the main barriers to the development of this field. Most of the compounds identified as having potential for therapy are structurally complex and therefore do not lend themselves to synthesis (Jensen and Fenical, 2000). Secondly, isolating these compounds from natural sources is not realistic, because active metabolites often make up a minute fraction of the organism's fresh weight (Fusetani, 2000). This scenario is similar to the difficulties involved in the development of the anticancer drug, taxol. Although technical advances may provide some solutions to supply issues, algae remain with the distinct advantage that mariculture techniques can be easily adapted to provide reliable and renewable culture of algae with therapeutic value (Jensen and Fenical, 2000).

MARINE ALGAL NATURAL PRODUCTS

Seaweeds have long been used in agriculture as manures to improve soil fertility (Chapman, 1970) as well as foliar sprays to nourish and reduce crop stress (Blunden and Gordon, 1986). Marine algae however do not have the ethnobotanical heritage of many land plants (Chapman, 1970). The first modern report of biological activity coming from algal metabolites dates back to 1940 when extracts of *Chlorella vulgaris* were found to have antibiotic properties (Pratt and Fong, 1940). It was almost twenty years later before any algal metabolites were found to be antiviral (Gerber *et al.*, 1958).

The emergence of viral chemotherapy is actually closely tied to marine natural product chemistry. The most successful class of both plant and animal antivirals, nucleoside analogs, are based on the discovery in the 1950s that arabinosides from sponges could be used therapeutically against viruses. Synthetic chemistry advanced this concept and led to the development of the two most successful antivirals: Ribavirin and Acyclovir. However only in the early eighties, with the beginning of systematic screening programs, were algae and other marine organisms screened comprehensively for antiviral activity (Rinehart *et al.*, 1981). Statistically metabolites from marine organisms are also the leading source for anticancer bioactivity (Munro *et al.*, 1987). Most research into the antiviral properties of marine algae has focused on members of the Rhodophyta and Heterokontphyta. Chlorophyta have not been closely evaluated for marine natural

products largely because their metabolites are unstable and most species are tropical (Carté, 1996; Paul and Fenical, 1987).

Intensive phytochemical studies of algal antiviral agents began with the identification of the human immunodeficiency virus (HIV) in 1984. Algal metabolites that have since been implicated in antiviral activity against human infection include: sulphated polysaccharides, polyphenols, peptides, and sulfoglycolipids (Schaeffer and Krylov, 2000). As the surveys of marine natural products have widened, bioassays have become more diverse (Munro *et al.*, 1987). Algal metabolites have been found to have bioactivity against human retroviruses, herpes viruses, togaviruses, paramyxovirues, rhabdoviruses (reviewed in Schaeffer and Krylov, 2000), poliovirus (Hudson *et al.*, 1999a,b) and plant tobamoviruses and necroviruses (Padmakumar and Ayyakkannu, 1997; Galal *et al.*, 1999).

POTATO VIRUS X

Algae screened for bioactivity in this study were tested against the *Potexvirus*, potato virus X, which is also known by the common names potato latent virus or potato mild mosaic virus. Particles are long flexous filaments with dimensions approximately 515 by 13 nm (Figure 2.1). The virus has a positive sense, single-stranded RNA genome and lacks a membrane envelope (Shaw, 1996). Symptoms in *Solanum tuberosum* depend on the strain but are generally mild, ranging from none to mild mosaic or necrotic streaking (Šutić *et al.*, 1999). The strain used in this study as a model for screening and for bioassay-guided fractionation was a severe strain, originally isolated in Prince Edward Island, Canada. There are no natural vectors of PVX; transmission occurs by vegetative propagation, contact between infected plants or mechanically. PVX occurs in potato growing areas around the world; however, in many areas the virus no longer presents a threat to production (Brunt *et al.*, 1996). PVX was chosen for this study because of viral stability and clear symptom development in indicator

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species. In nature the virus mainly infects members of the Solanaceae, however it can also be successfully replicated in a number of other hosts from the following genera: *Amaranthus* (Amaranthaceae), *Brassica* (Brassicaceae), *Chenopodium* (Chenopodiaceae), *Euphorbia* (Euphorbiaceae), *Gomphrena* (Amaranthaceae), *Trifolium* (Fabaceae) and *Vitis* (Vitaceae) (Šutić *et al.*, 1999).



Figure 2.1: Transmission electron micrograph of potato virus X particles. Virus particles are long flexuous rods approximately 13 x 515 nm (Source: University of Columbia in New York City, 2001).

BIOASSAYS

It is important in the design of a screening program to choose bioassays that reliably reconstruct the conditions of the infection to be treated. Fortunately when testing for agents against plant viruses, ethical constraints do not limit assays either *in vivo* or in the intended host. This provides some reassurance that antiviral compounds in the assay will have relevant activity within the host (Munro *et al.*, 1987). The two bioassays used in this study were the local lesion assay and systemically infected potato tissue culture, which tested bioactivity of compounds against local and systemic infections, respectively. The latter assay was used to assess the most antiviral algal extract, which was obtained from *Fucus gardneri*, against established viral infection in *S. tuberosum*. Since *S. tuberosum* does not exhibit a hypersensitive response to PVX, infection is spread systemically producing no quantifiable lesions. Therefore the ELISA was used to

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detect and assay virus in the host. The design of this experiment was intended to reflect plant virus chemotherapy methods used by industry so that efficacy, absorption and toxicity of the extract could be more fully assessed.

Local lesion assays were invaluable to the project, allowing differentiation between viable and non-viable virus in the screening of algal extracts for bioactivity. *Chenopodium quinoa* was used as the host because of the clear countable lesions it produces in response to infective PVX particles. This assay relies on the hypersensitive response of such an indicator species to create foliar lesions in response to pathogen infection (Matthews, 1991). Two actions characterize a response. One is the necrosis of cells at and surrounding the entry point of the virus, this is the response that allows virologists to quantify virus infectivity (Figure 2.2). The other is a dramatic change in metabolism of surrounding cells, such as the creation of mechanical barriers, defense proteins and phytoalexins. These changes help to contain the spread of the virus by contributing to resistance in surrounding cells (Fritig *et al.*, 1990). Local lesion assays can discern a difference in concentration of viable virus with a resolution of 10-20% (Matthews, 1991).



Figure 2.2: Lesion development on *Chenopodium quinoa* in local lesion assay. Treatment side (left) shows complete inhibition of PVX infectivity, while development of lesions on control side (right) indicates infective virus particles.

MARINE ALGAE

As primary producers, algae are extremely important to the global energy balance, contributing more than any other group to the world's biomass (Lee, 1999). Taxonomically they are a large and difficult to define group of organisms. This complexity comes from the fact that algae are polyphyletic; that is, they do not share a single common ancestor. The grouping is instead based on a shared form and function (Graham and Wilcox, 2000). Algae are thallophytes, plants without stems, leaves and roots, containing chlorophyll *a* (Lee, 1999). Algae range in size from single cell diatoms to massive kelps over 40m in length (Witvrouw and De Clercq, 1997). Most algae, including all in this study, are found in aquatic environments (Lee, 1999). Depending on the taxonomic scheme used to classify algae, they can be divided into eight or nine phyla. Traditionally this was done using accessory photosynthetic pigments and cell structure (Table 2.1) (Bold and Wynne, 1985). Recently, molecular techniques have essentially corroborated the existing classification, with only a few minor changes to the phylogenetic organization (Table 2.2) (Lee, 1999).



Table 2.1: Major classical algal phyla. Bold text refers to taxa that have since been grouped. (Adapted from Bold and Wynne, 1985)
Heterokontophyta (Chrysophyceae, Synurophyceae,		
Dictyochophyceae, Pelagophyceae, Bacillariophyceae,		
Xanthophyceae, Eustigmatophyceae, Phaeophyceae)		Seaweeds
Rhodophyta (red algae)		
Chlorophyta (green algae)		
Prymnesiophyta (Chrysophyta)		
Euglenophyta (euglenoids)	-	Micro-algae
Dinophyta (was Pyrrophyta)		
Glaucophyta (chloroplasts are endosymbiotic		
cyanobacteria cyanelle)		
Cryptophyta (cryptomonads)		
Cyanophyta (cyanobacteria)		

Table 2.2: Algal phyla based on classical taxonomy and molecular sequence information. Bold text refers to newly formed taxa. (Adapted from Lee, 1999)

Based on the more recent classification, this study evaluated the bioactivity of algae from the Heterokontophyta, Rhodophyta and Chlorophyta (Table 2.2). Heterokont species evaluated in the screening belong to the brown algae and diatoms. This group is fundamentally different from the other algal divisions and consequently is classified in the Kingdom Chromista. Both Rhodophyta and Chlorophyta belong to the Kingdom Plantae. Specifically, members of the Heterokontophyta are distinguished from other algae by the presence of two heteromorphic flagellae, plastids encased with a fold of endoplasmic reticulum, chlorophyll c but never b, and carotenoids such as fucoxanthin (Graham and Wilcox, 2000). Diatoms are strictly unicellular or colonial and are common in both fresh and salt water. The most notable feature of the diatoms is the siliceous frustule. This detailed and delicate covering protects the cells and provides key diagnostic features for identification (Van Den Hoek *et al.*, 1995). In contrast, brown algae (Phaeophyceae) have no unicellular or

colonial forms and reside almost exclusively in marine environments (Lee, 1999). The dark brown colour comes from accumulations of tannins and fucoxanthin that mask chlorophyll (O'Clair and Lindstrom, 2000). Members of the brown algae are dominant in the cold waters of the Northern and Southern Hemispheres such as coastal British Columbia, and are found abundantly in the intertidal and subtidal zones (Lee, 1999).

The Rhodophyta are more closely related to higher plants than brown algae and are also mostly found in marine environments (O'Clair and Lindstrom, 2000). Their chemistry has been the most widely studied of the marine algae because of the abundance of halogenated compounds with high bioactivity (Carté, 1996). Although often red in color, varying amounts of the three primary pigments green chlorophyll, red phycoerythrin and blue phycocyanin lead to a wide range of coloration. These accessory pigments allow red algae to absorb green and blue light, thus permitting these algae to inhabit deeper reaches of the water column (O'Clair and Lindstrom, 2000). Red algae exist as a range of growth forms, from single cells or filaments to complex filamentous aggregations. As with the red algae *Lithothamnium* sp. and *Corallina officinalis* that were screened for PVX inhibition, the cell walls of some red algae are calcified. These particular red algae are called corallines and are especially common in warmer waters (Graham and Wilcox, 2000).

Green algae exist as either unicells or multicellular forms. As the name suggests the Chlorophyta are distinguished by the dominance of the bright green pigment chlorophyll. The Chlorophyta are the most diverse phylum of algae and most of its species (87%) are found outside of marine environments. Evidence suggests that green algae (Charophycaceae) are the ancestors to modern land plants. Shared characters between green algae and higher plants include: chlorophyll pigments *a* and *b*, α and β carotenes, starch storage and in some cases cellulose. (O'Clair and Lindstrom, 2000). Below are descriptions of the taxonomy, habitat and structure for each of the species included in this study (Algaebase, 2001, O'Clair and Lindstrom, 2000; Hustedt, 1985; Scagel, 1967). The lifecycles of marine algae are complex and involve alternating generations between gametophyte and sporophyte. Since no general rules apply and some lifestages look identical while others are radically different, each species description is accompanied with a sketch.

HETEROKONTOPHYTA

Alaria nana Schrader Order: Laminariales Family: Alariaceae Distribution: Cape Yakataga, Alaska to Oregon on exposed coasts Bathymetry: mid-low intertidal Description: A golden brown to olive color. Holdfast is large and with many rootlike haptera. Stipe is thick and cylindrical. Strap-shaped sporophylls just above holdfast. Above this point, stipe gives rise to conspicuous vegetative blade. Blade is up to 3 m long.



Colpomenia peregrina (Sauvageau) Hamel Order: Ectocarpales Family: Scytosiphonaceae Distribution: Alaska to central California, North Atlantic, Australia, New Zealand Bathymetry: low intertidal Description: Yellow brown color. Globular, thin walled sacs. Tapered at base, no stipe and small holdfast.



Costaria costata (C. Agardh) Saunders Order: Laminariales Family: Laminariaceae Distribution: Unalaska Island, Alaska to southern California, Japan and Russia. Bathymetry: low intertidal and upper subtidal Description: Yellow-brown to brown in color. Holdfast is branched and leads to a stipe with 5-7 parallel ribs. These ribs continue as the stipe widens into the blade that tapers at the top. Between ribs the blade is bullate. 15 cm



KР

Cymathere triplicata (Postels & Ruprecht) J. Agardh Order: Laminariales Family: Laminariaceae

Distribution: Bering Sea to Northern Washington. Kurile Islands, Russia.

Bathymetry: low intertidal to upper subtidal.

Description: Yellow-brown in color. The holdfast is discoid and without branching. The stipe is short and leads to 3 ribs running the length of the undivided blade. Has a cucumber-like smell.

Egregia menziesii (Turner) Areschoug

Order: Laminariales

Family: Lessoniaceae

Distribution: Queen Charlotte Islands to Mexico. Bathymetry: intertidal to shallow subtidal

Description: Dark brown to olive in color. Holdfast is branched and leads to a flattened stipe covered with papillae. It is branched irregularly with the sides of the branches being fringed with small blades and air bladders. (common name is feather boa kelp)



Fragilaria oceanica Cleve

Order: Fragilariales Family: Fragilariaceae Distribution: Northern Hemisphere Bathymetry: mid to upper intertidal Description: Colonial, forming dense ribbons of unicells. In valve view frustule is lanceolate 8-60µm long, 3-8 µm wide with delicate transapical straie. In girdle view rectangular and linear.



Fucus gardneri Silva

Order: Fucales Family: Fucaceae Distribution: Bering Sea, Alaska to central California, Kamchatka Bathymetry: upper to mid intertidal Description: Brown to brown-olive color. Is often dominant in the mid intertidal. The holdfast is discoid and leads to flat, dichotomously branched blades. Blades have percurrent midrib throughout. At the tips of blades are receptacles containing gametes.



Hedophyllum sessile (C. Agardh) Setchell Order: Laminariales

Family: Laminariaceae Distribution: Aleutian Island, Alaska to Monterey

Country, California. Bathymetry: mid to low intertidal Description: Dark brown and prefers wave exposure. Very abundant at Whiffin spit collection site. The holdfast is finely divided and leads directly to the blade, with no stipe being present (very short stipe is present when young). The blade is fan shaped and has a pitted texture. As the season passes the blade becomes very torn.



Laminaria saccharina (Linnaeus) J. V. Lamouroux

Order: Laminariales

Family: Laminariaceae

Distribution: Aleutian Islands, Alaska to California; Japan; Arctic and North Atlantic.

Bathymetry: low intertidal to upper subtidal

Description: Brown to brown-olive in color. The conspicuous holdfast is branched and leads to a cylindrical stipe. The blade is smooth with slightly ruffled edges.



Nereocystis luetkeana (Mertens) Postels & Ruprecht

Order: Laminariales Family: Lessoniaceae Distribution: Aleutian Islands, Alaska to California Bathymetry: Subtidal to 17m Description: Brown in color. Is one of the largest brown algae. The holdfast is branched and can be as wide as 40 cm in diameter. The stipe makes up most of *Nereocystis* with the average length being about 10m. The upper end of the stipe is hollow and forms a tapered float. From the top of the bulb, two groups of blades (as many as 20) emerge. Often form thick beds known as kelp forests.



Ralfsia sp. Berkeley Order: Ralfsiales Family: Ralfsiaceae Distribution: Aleutian Islands, Alaska to Humbolt County, California; Arctic, North Atlantic Bathymetry: middle to low intertidal Description: Crustose algae, forming olive-brown circular patches 1-2 mm thick. Broadly overlapping lobes. Slow growing.



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Soranthera ulvoidea Postels & Ruprecht Order: Ectocarpales Family: Punctariaceae Distribution: Alaska to California Bathymetry: mid intertidal Description: Have a balloon-like shape and are covered with evenly distributed brown bumps. Tapered at the base. Inconspicuous discoid holdfast. Epiphytic on Neorhodomela and Odonthalia.



RHODOPHYTA

Bossiella orbigniana (Decaisne) Silva Order: Corallinales Family: Corallinaceae Distribution: northern Southeast Alaska to Mexico; Argentina

Bathymetry: extreme low intertidal and subtidal Description: Combination of red pigments and calcium carbonate give this alga a pink color. Both the crustose base and erect branches it gives rise to are calcified. Branches are pinnately arranged off "wingnut" shaped segments greater than 2 - 3 mm across and about 1 mm long.

Clathromorphum reclinatum (Foslie) W.H.Adey

Order: Corallinales Family: Corallinaceae Distribution: Alaska to southern California Bathymetry: low intertidal Description: Epiphytic, a combination of red pigments and calcium carbonate give this alga a pink color. Is slightly umbrella shaped with undulations. A close look reveals concentric circles on the disk.





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Corallina officinalis var. *chilensis* (Decaisne) Kützing

Order: Corallinales Family: Corallinaceae Distribution: Prince William Sound, Alaska to Mexico and Chile; Japan; Russia Bathymetry: low intertidal and subtidal Description: Combination of red pigments and calcium carbonate give this alga a pink color. Both the crustose base and erect branches are calcified. Branches are pinnately arranged at each of the 1.5 to 3 mm segments of the axis. Segments of the axis are flattened cylinders.



Halosaccion glandiforme (S.G. Gmelin) Ruprecht Order: Palmariales

Diuel. Faimanaies

Family: Palmariaceae

Distribution: Bering Sea, Alaska to Point Conception, California; Kamchatka.

Bathymetry: mid intertidal

Description: Ranges in color from purple/red to yellow green depending on season. Elliptically shaped sacs are erect and filled with water. Attached at base by narrow discoid holdfast.



Hildenbrandia sp. Nardo

Order: Hildenbrandiales Family: Hildenbrandiaceae Distribution: Aleutian Islands, Alaska to Panama; Arctic; Chukchi sea; Galapagos Islands; Mediterranean, North Atlantic, Baltic. Bathymetry: high to mid intertidal Description: Deep red color. A fleshy crustose alga closely (<1mm) adhered to rocks. Only sporophytic phase has been found, no gametophytic phase known. Slow growing.



КP

Lithothamniom sp. Heydrich Order: Corallinales Family: Corallinaceae Distribution: Alaska to California; Galapagos Islands, Ecuador and Japan. Bathymetry: low intertidal on rocks and mid intertidal in tidal pools (intolerant to desiccation) Description: Pink calcified crusts (~1mm) covering rocks and mollusks. Often with small branches reaching up to 6 mm from the surface.



(O'Clair and Lindstrom, 2001)

Mastocarpus papillatus (C. Agardh) Kützing (Gametophyte) Order: Gigartinales Family: Phyllophoraceae Distribution: Bering Sea, Alaska to Mexico; Japan and Russia Bathymetry: mid upper intertidal

Description: A rich deep purple/red. On a short stipe, gametophytic blades annually grow out of a perennial crustose base. Blades are dichotomously branched and are covered with gamete containing papillae, giving the surface a rough texture.



Mastocarpus papillatus (C. Agardh) Kützing (tetrasporophyte)

(formerly *Petrocelis franciscana*) Order: Gigartinales Family: Phyllophoraceae Distribution: Bering Sea, Alaska to Mexico; Japan and Russia Bathymetry: mid upper intertidal Description: Produced by gametophyte carpospores. Due to their heteromorphic nature, the tetrasporophytes were previously thought to be another species. Tetrasporophytes are also known as sea tar because of their black lobed crust. The crust is fleshy with irregular edges. Slow growing.



(O'Clair and Lindstrom, 2000)

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Mazzaella splendens (Setchell & N.L. Gardner) Fredericq Order: Gigartinales Family: Gigartinaceae Distribution: Gulf of Alaska to Mexico Bathymetry: low intertidal to upper subtidal Description: Dark purple in color. A narrow holdfast leads to a short stipe and single blade. The ovate blade is thick and ruffled at the edges.



Microcladia borealis Ruprecht Order: Ceramiales Family: Ceramiaceae Distribution: Aleutian Islands, Alaska to San Luis Obispo County, California Bathymetry: mid to low intertidal Description: Dark red to olive in color. The holdfast is made up of a prostrate branch that is slightly dissected. Alga is erect and branching in one plane, the blades are finely divided up to six times. Branches are arched and arise from the inside of the previous blade.



Odonthalia floccosa (Esper) Falkenberg Order: Ceramiales Family: Rhodmelaceae Distribution: Bering Sea to Santa Barbara, California

Bathymetry: low intertidal

Description: Dark red to dark brown. Called the sea brush, this alga is highly branched. The main axis is erect and undulates.



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Porphyra perforata J. Agardh

Order: Bangiales Family: Bangiaceae Distribution: Aleutian Islands, Alaska to Mexico. Okhotsk Sea, Russia. Bathymetry: upper to low intertidal Description: Is purple/red in color. Holdfast is discoid and leads to a round ruffled blade.



Prionitis lyallii Harvey Order: Halymeniales Family: Halymeniaceae Distribution: Esquimalt, British Columbia to Baja, California Bathymetry: low intertidal to subtidal Description: Brown to bright brick red in color. Small holdfast leads to stipe (1-7 cm long) that is divided dichotomously several times. Blades are lanceolate and 3-45 cm long. A diagnostic feature of this alga is a distinct chlorine odor.



Smithora naiadum (Anderson) Hollenberg Order: Compsogonales Family: Erythrotrichiaceae Distribution: Kodiak Island, Alaska to Mexico Bathymetry: low intertidal to upper subtidal Description: Small red epiphyte of *Phyllospadix* and *Zostera* grasses. Cushion shaped holdfasts attach to the surface (margins) of the grass. Blades are thin and taper to a short stalk at the base. Sketch is of *Smithora naiadum* living epiphytically on *Phyllospadix scouleri*.

KР

Chlorophyta

Acrosiphonia coalita (Ruprecht) Scagel, Garbary, Golden & Hawkes (new name Acrosiphonia mertensii) Order: Ulotrichales Family: Acrosiphoniaceae Distribution: Aleutian Islands, Alaska to central California Bathymetry: mid to high intertidal Description: Gametophyte is a green filamentous alga up to 25 cm in length. The ends of the filaments have diagnostic hooks. The sporophyte phase lives endophytically in

Mastocarpus papillatus which is included in this collection.

Codium fragile (Suringar) Hariot Order: Codiales Family: Codiaceae Distribution: Mexico to Alaska, Japan and introduced to Atlantic Bathymetry: mid intertidal to subtidal Description: Dark blackish green in color. Tubular branches that are repeatedly dichotomously branched. Spongy and felt-like in texture.



Ulva fenestrata Postels & Ruprecht Order: Ulotrichales Family: Ulvaceae Distribution: Alaska to Southern California Bathymetry: mid to low intertidal Description: Bright green, perforated, sheet-like blade arising from a holdfast.



Ulva taeniata (Setchell ex Collins) Setchell & N.L. Gardner Order: Ulotrichales Family: Ulvaceae Distribution: Southern British Columbia to Monterey County, California Bathymetry: mid to low intertidal Description: Blades are bright green. The holdfast is discoid and gives rise to one or several long blades (up to 1.5m). Edges of the blades are spirally twisted and slightly ruffled.



ΑΝΤΗΟΡΗΥΤΑ

Phyllospadix scouleri J. D. Hooker

Family: Potamogetonaceae Distribution: Sitka, Alaska to Mexico Bathymetry: upper subtidal Description: Although it appears to be a grass, *Phyllospadix* is not from the Poaceae. This surf grass is



specially adapted to survive marine environments. Adaptations include: tolerance of high salt, flexible leaves and non-lignified xylem to withstand heavy surf, air spaces for floatation and complete lack of stomata. These plants are usually less than 1m and leaves 4mm wide. Well-developed roots and inconspicuous flowers which yield small heart-shaped fruit. Sketch is of *Phyllospadix scouleri* with *Smithora naiadum* epiphyte.

Methods and Materials

COLLECTION

Marine algae were collected at several locations including: French Beach, Vancouver Island (48° 23' N, 123° 56' W), Acadia Beach, Vancouver (49° 17' N, 123° 14' W) and Towers Beach, Vancouver (49° 16' N, 123° 15' W). The primary collection was done at Whiffin Spit, Vancouver Island, (48° 22' N, 123° 43' W) on July 28, 1999, during a –1.5 tide (Figure 2.3). At least three specimens were collected for each species. Samples were rinsed with fresh tap water to remove debris and epiphytes, air-dried at room temperature and stored at 4°C. For each collection a voucher specimen was prepared and deposited at the University of British Columbia herbarium (UBC A84379-A84352). Vouchers were identified to species using Scagel (1967); Hustedt, (1985) and O'Clair and Lindstrom (2000) and classification was confirmed with the help of Drs. Rob DeWreede, Sandra Lindstrom and Max Taylor (Botany Department, University of British Columbia).



Figure 2.3. Collection site Whiffin Spit, Vancouver Island. a) low to mid intertidal b) upper intertidal.



All of the taxa screened in this study, except the sea grass *Phyllospadix scouleri*, were algae. Since species composition changes significantly throughout the littoral zone, collections were made from low to high intertidal. Causes for these changes in community diversity relate to species' adaptation to length of exposure between tides and aquatic light conditions (Levring, 1979). In choosing the collection site, algal diversity was an important consideration. Whiffin Spit on southwestern Vancouver Island has moderate wave exposure and salinity as well as an intertidal zone of several hundred metres. The moderate growing conditions combined with a large habitat allow for wide range of species to inhabit Whiffin Spit, thus contributing to biodiversity (R.E. DeWreede personal communication) which in turn is often associated with chemical diversity (Carté, 1996).

SOURCE OF VIRUS

A severe strain of PVX from collections at the Pacific Agri-Food Research Centre (Summerland, B.C., Canada) was purified and stored as previously described (French & Towers, 1992).

EXTRACTION

Approximately 2g of each algal specimen was ground in liquid nitrogen using a mortar and pestle and extracted three times with 30ml of 100% methanol (ACS grade- Fisher). Grinding with liquid nitrogen was the best way to overcome the tough, elastic nature of the algae. Resulting supernatants were vacuum filtered (Whatman No.1 filter paper) and dried *in vacuo* at 30 °C using a Yamato model RE 47 rotavaporator (Japan).

BIOASSAYS

Local lesion assay

Extracts were tested for antiviral activity against PVX using local lesion assays (LLA) in C. quinoa (French et al., 1991). Screening of each algal extract for anti-PVX activity was done using three plants (5-6 weeks of age), each with 4 test leaves. Control and treatment replicates were applied to the same leaf by dividing the 4 test leaves per plant arbitrarily into control and treatment halves. To compare the effect of one sample over another on the infectivity of the virus, it is recommended that at least six to eight leaf halves be tested. In this study all extracts were tested on 12 - 24 leaf halves (Matthews, 1991). Leaves were dusted with carborundum, a fine abrasive that facilitates virus transmission by producing small openings in the epidermal layer. The control side of each leaf received 575ng of PVX (severe strain) in 10µl of control solution (5% aqueous 2-methoxy ethanol-Fisher). Each treatment side also received 575ng of PVX suspended in 10μ l of control solution, plus the respective algal extract at a final concentration of $10\mu g/\mu l$. Both the control and treatment virus preparations were incubated (25°C water bath/shaker) for 30 minutes before application to leaves with a gloved finger. Infected plants were placed in a growth chamber (20°C, cool white light, 16h light:8h dark) for 5 to 6 days while lesions developed. The effect of the algal extract on viral infectivity was determined by comparing the relative number of lesions on both sides of the test leaves. All extractions and assays were completed in duplicate using the above methods.

The activity of selected extracts was determined by testing four crude extracts of moderate to high antiviral activity (*E. menziesii*, *A. nana*, *U.taeniata*, and *F.gardneri*) in a dilution series $(10\mu g/\mu l, 1\mu g/\mu l, 0.1\mu g/\mu l$ and $0.01\mu g/\mu l$) using the above method. These four extracts plus *Fragilaria oceanica* were further tested $(10\mu g/\mu l)$ four months after their extraction to evaluate the stability of their active constituents. To complement the LLAs with PVX, assays were repeated with tobacco mosaic virus (TMV) and potato virus Y (PVY). Difficulty with PVY infectivity resulted in assays being repeated with virus from several sources and from different preparations. These included testing 14 PVY isolates (Table 2.3) from Pacific Agri-Food Research Centre frozen storage in hosts: *C. quinoa*, *C. quinoa* cv. 'sensitive' and/or *Nicotiana alata* cv. 'lime green'.

Table 2.3: Potato virus Y isolates inoculated into all or one of the following hosts, *C. quinoa*, *C. quinoa* cv. 'sensitive', *Nicotiana alata* Cv. 'lime green'.

Potato virus Y isolates from PARC			
PVY 37 B (14/5/93)			
PVY 37 C (18/5/93)			
PVY 37 D (8/8/93)			
PVY (24/5/83)			
PVY F batch I (4/1/74)			
PVY F batch II $(4/1/74)$			
PVY ⁰⁻³			

Application of *Fucus gardneri* extract to PVX infected potato tissue culture

Crude and semi-pure fractions of *F. gardneri* were assayed for antiviral activity against PVX using systemically infected cultures of *S. tuberosum* cv. "True Blue". Sterile cultures of *S. tuberosum* were grown on solid medium (30g sucrose, 4.4g BC potato media 82 (PM) (Carolina Biological Supply), 5.6g agar/litre, pH 5.7) and leaves were mechanically inoculated with 10µl of PVX (1.15mg/ml) in 10mM sodium phosphate buffer, pH 7.2. After 21 days, PVX infection was confirmed by ELISA. Stems were severed leaving five nodes. After removing the leaves, ten shoots were transferred aseptically to liquid culture (PM without agar) in two Sigma culture boxes. Liquid media were modified by the addition of algal extracts to produce six treatments: crude *F. gardneri* (10µg/µl, 1µg/µl & 0.1µg/µl), semi-pure *F. gardneri* 1µg/µl (butanol fraction), positive control (Ribavirin 0.02µg/µl) and negative control (no treatment). Plant cultures were grown in a Sanyo growth chamber (16h light: 8h dark/ 23°C). At 30 days,

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shoot tips were excised from the developed lateral shoots and transferred to solid media containing either treatments as before or fresh standard media without additions. At sixty days, 0.05 - 0.15g of leaves from each treatment were collected and ground (Erich Pollänhne, model R30DT71K4/2 with beveled rollers manufacturer, Germany) with extraction buffer [8.0g NaCl, 0.2g KH₂PO₄, 1.15g Na₂HPO₄, 0.2g KCl, 2g polyvinyl-pyrrolidone (M.W. 44,000), 0.5ml Tween 20/litre, pH 7.4] to a final concentration of 0.15g/ml. Plant homogenates were assayed for PVX using ELISA (Clark *et al.*, 1976).

In preparation for ELISA, 96 well polystyrene trays were coated with PVX-specific antibodies dissolved in 100µl of coating buffer [1.82g Na₂CO₃·H₂O, 2.93g NaHCO₃/litre with 2µl PVX-specific antibody (final antibody concentration 1/5000 stock - Phytodiagnostics, North Saanich, B.C., Canada) per 10ml buffer] per well. Trays were incubated at 37°C for 4 hours, washed three times using 100-200µl PBS-T buffer (8.0g NaCl, 0.2g KH₂PO₄, 1.15g Na₂HPO₄, 0.2g KCl, 0.5ml Tween 20/litre, pH 7.4) per well and blotted dry. Sample homogenates (100µl/well) and standards in duplicate wells were incubated at 4°C overnight, washed and blotted dry. Conjugate buffer (100µl/well) [0.2% BSA and 10µl conjugate antibody (final antibody concentration 1/1000 stock solution -Phytodiagnostics, North Saanich, B.C., Canada) per 10ml of PBS-T buffer was added and the trays were incubated for 2 hours at 37 °C. To ensure all unbound conjugate antibody was removed, wells were washed 4 times with PBS-T buffer and blotted dry. Next added 100µl per well of enzyme substrate 15mg pnitrophenyl phosphate (Sigma) to 30ml substrate buffer (diethanolamine 97ml per litre pH 9.8)]. The resulting color reaction is proportionate to the virus titer of each well. Absorbance was monitored (405nm) with an ELISA plate reader (Titertek Multiskan, Flow Laboratories International, Switzerland) at 20, 40 and 60 minutes.

Results

LOCAL LESION ASSAYS

Initial screening of algal extracts showed antiviral activity with two (*Ralfsia* sp. and *Codium fragile*) of the five extracts inhibiting PVX infectivity by more than 90%. Of the 31 species tested, extracts of algae collected had effects ranging from slight stimulation (19%) to complete inhibition of PVX infection (Table 2.4). Six species (*F. gardneri, A. nana, Ralfsia* sp., *E. menziesii, C. fragile* and *Fr. oceanica*.) had strong (>80%) antiviral effects. When applied to the leaves of *C. quinoa* none of the extracts appeared to cause obvious phytotoxicity such as necrosis, yellowing or reduced growth. When tested against TMV in *C. quinoa*, only the extract of *F. gardneri* resulted in viral inhibition greater than 80% (Table 2.5). Unfortunately due to problems with greenhouse pests, not all algal extracts were successfully assayed for activity against TMV. Problems with stability of PVY infectivity following virus isolation resulted in no data being collected for this virus.

Chapter 2: Results

Species	% Inhibition of DVX infectivity
Heterokontonhyta	ΤνΑιμεεινη
Eucon conducti	100.0+0
rucus garaneri Alavia nana	98 9+0 4
Ralfeia en *	97.2+0.7
Fragilaria oceanica	83 5+10 6
Fraguaria occanica Faregia menziesii	80 3+2 5
Colnomenia pereorina	50.0 ± 17.1
Hedonhvllum sessile	44.6±10.6
Cymathere triplicata	39.8±17.4
Laminaria saccharina	38.8±18.6
Nereocvstis luetkeana	31.3±18.4
Costaria costata	27.5±12.7
Soranthera ulvoidea	26.19±24.5
Chlorophyta	
Codium fragile*	92.8±3.4
Ulva taeniata	77.1±25.6
Ulva fenestrata	73.8±25.2
Acrosiphonia coalita	44.9±10.7
Rhodophyta	
Porphyra perforata	45.7±9.8
Smithora naiadum	44.7±11.6
Mazzaella splendens	41.2±14.6
Clathromorphum reclinatum	37.3±13.2
Bossiella orbigniana*	36.4±13.6
Prionitis lyallii	29.7±20.8
Hildenbrandia sp.	24.4±26.1
Microcladia borealis	23.2±25.0
Corallina officinalis*	19.5±3.2
Mastocarpus papillatus (gametophyte)	18.8±21.9
Odonthalia floccosa	7.13±15.4
Mastocarpus papillatus (tetrasporophyte)	7.0±22.5
Lithothamniom sp.	-0.9±28.7
Halosaccion glandiforme	-19.7±29.4
Angiosperm	
Phyllospadix scouleri	20.0±8.9

Table 2.4: Local lesion antiviral assay from algae screening. Inhibition of potato virus X infectivity by extracts of marine organisms at $10\mu g/\mu l$ using a local lesion assay on the leaves of *C. quinoa*.

*Indicates the 5 algal extracts tested in initial screening. Results are the average of two separate experiments (standard error represented with \pm), each experiment consisting of 3 plants with 4 test leaves. Positive values refer to viral inhibition. Negative values refer to viral stimulation.

Extract	% Inhibition of
	TMV-µ1
Fucus gardneri	86.2±4.9
Ralfsia sp.	59.6±7.3
Fragilaria oceanica	46.8±11.4
Microcladia borealis	43.4±27.9
Ulva fenestrata	41.1±8.0
Ulva taeniata	29.2±18.7
Codium fragile	24.5±17.5
Hedophyllum sessile	20.5±36.7
Porphyra perforata	18.8±21.3
Alaria nana	- 4.1±2.9
Mazzaella splendens	- 5.2±32.8
Costaria costata	-34.6±19.2

Table 2.5: Inhibition of TMV-µ1 by select algal extracts. Antiviral effect determined using local lesion assays on *C. quinoa*.

Results are means \pm standard error of two separate experiments, each experiment consisting of 3 plants with 4 test leaves. Positive values refer to viral inhibition. Negative values refer to viral stimulation.

Results from the dilution series $(10\mu g/\mu l - 0.001\mu g/\mu l)$ showed the effect of concentration on the bioactivity of select extracts (Table 2.6). Full strength treatments $(10\mu g/\mu l)$ were consistent with results found in previous screening. At a concentration of $1\mu g/\mu l$, *F. gardneri* was the only extract to inhibit PVX infection by more than 80%. As algal extract concentration was reduced many treatments stimulated virus infectivity. The five extracts assayed four months after their extraction resulted in quite different apparent stabilities of bioactive molecules (Table 2.7). Extracts from both *Fr. oceanica* and *U. taeniata* showed a dramatic loss of bioactivity, whereas the bioactivity of the other three extracts remained essentially unchanged.

Table 2.6: Percent PVX inhibition tested, using a local lesion assay on C. quinoa, over a logarithmic dilution series of four crude extracts.

Concentration of dilution series						
Species	10µg/µl	1µg/µl	0.1µg/µl	0.01µg/µl	0.001µg/µl	
Fucus gardneri	99.5±0.4	93.6±2.5	-2.1±10.3	-59.5±21.2	-16.8±13.8	
Alaria nana	99.4±0.3	24±21.7	-4.1±55.9	5.6±7.4	ND	
Egregia menziesii	85.9±1.1	-29±41.9	-54.2±20	-12±27.8	ND	
Ulva taeniata	73.5±7.9	16.7±14	-93±27.6	-52.1±55.4	18.7±13.4	

Results are means ±standard error of two separate experiments, each experiment consisting of 3 plants with 4 test leaves. Positive values refer to viral inhibition. Negative values refer to viral stimulation. ND: not determined.

Table 2.7: Anti-PVX activity of select algal extracts 4 months after extraction.

Species	% Inhibition of PVX infectivity	Original Bioactivity
Alaria nana	98.9±0.7	98.9±0.4
Egregia menziesii	81.8±8.9	80.3±2.5
Fragilaria oceanica	36.4±12.8	83.5±7.3
Fucus gardneri	100 ± 0	100±0
Ulva taeniata	-22.8 ± 4.9	77.1±25.6

Results are means ±standard error of two separate experiments, each experiment consisting of 3 plants with 4 test leaves. Positive values refer to viral inhibition. Negative values refer to viral stimulation.

APPLICATION OF F. gardneri EXTRACT TO PVX INFECTED POTATO TISSUE CULTURE

After 30 and 60 days of chemotherapy, algal treatments showed little or no reduction in PVX concentration, whereas ribavirin showed a considerable antiviral effect (Table 2.8). Inclusion of 30 day and 60 day chemotherapy exposures was designed to verify virus eradication rather than virus suppression. Virus suppression would result in 30 day treatments showing increased virus titer after removal from chemotherapy. In treatments that were antiviral this was not the case, all 30 and 60 day treatments were comparable. Ribavirin treatment (**E**) resulted in general stunting as found previously (Wambugu *et al.*, 1985) (Figure 2.4). The highest concentration of crude *F. gardneri* (**C**) was severely phytotoxic leaving stems etiolated, leaves reduced in number and size and limited root development.

Table 2.8: Effects of crude and semi-pure fractions of *Fucus gardneri* extract were tested against PVX-infected *S. tuberosum* culture.

	Absorbance		
Ireatment	30 days*	60 days*	
Crude Fucus extract @ 10µg/µl (C)	0.556 ± 0.024	ND	
Crude Fucus extract (a) $1\mu g/\mu l$ (B)	0557 ± 0.012	0.455 ± 0.020	
Crude Fucus extract (a) $0.1 \mu g/\mu l$ (A)	0.553 ± 0.012	0.442 ± 0.045	
Semi-pure Fucus extract @ 1µg/µl (D)	0.573±0.009	0.496 ± 0.009	
Negative control (no treatment)	0.382 ± 0.077	0.510 ± 0.008	
Positive control (Ribavirin @ 20µg/ml) (E)	0.116 ± 0.104	0.009±0.001	

^aAbsorbance at 405nm measures color change in ELISA assay, and consequently is proportional to PVX concentration in the sample.

Two treatments: 30 days chemotherapy followed by 30 days on unmodified media, 60 days chemotherapy. ND: not determined due to toxicity. Each treatment measurement is the summary of 5-10 replicates.



Figure 2.4: Potato tissue culture after 30 days of chemotherapy. Liquid media of each treatment supplemented (A) crude *Fucus gardneri* 0.1mg/ml (B) crude *Fucus gardneri* 1mg/ml (C) crude *Fucus gardneri* 10mg/ml (D) *Fucus gardneri* butanol fraction 1mg/ml (E) Ribavirin 20µg/ml. Reduced growth due to toxicity of treatments in E and particularly C.

Discussion

LOCAL LESION ASSAY

Marine algae have only recently been found to contain compounds inhibitory to plant viruses (Padmakumar and Ayyakkannu, 1997; Galal et al., 1999). This current study adds to the list of taxa known to contain such compounds and is the first to screen for activity against PVX. Local lesion assays detected inhibition of infectivity suggesting an antiviral effect occurring either in vitro during incubation or in vivo during the onset of infection. Of 31 species screened, six contained strong bioactivity (>80%) that dramatically reduced the number of local lesions induced on C. quinoa (Table 2.4). Finding that 20% of extracts strongly inhibit viral infectivity adds credence to previous claims that marine extracts have great potential therapeutic value (Munro et al., 1987; Carté, 1996). Extracts of H. glandiforme (19% stimulation) and F. gardneri (100%) inhibition) represent the extremes of antiviral effect found in the screening. To delineate the potency of some of the most active extracts, F. gardneri, A. nana, E. menziesii and U. taeniata were assayed over a concentration gradient (Table 2.6). The antiviral performance of crude F. gardneri extract at $1\mu g/\mu l$ emphasizes the potential of this extract for phytochemical analysis. Below a concentration of $1\mu g/\mu l$, several treatments resulted in stimulation of viral infection. This effect may be an artifact of experimental design. However, it possibly illustrates concentration dependent interactions between viral antagonizers and stimulators (i.e.: RNase inhibitors) both present in the algae.

These four extracts plus *Fr. oceanica* were tested 4 months after extraction to evaluate the stability of the bioactive compounds. The extracts of all three brown algae (*A. nana, E. menziesii, F. gardneri*) remained unchanged, whereas both *U. taeniata* (Chlorophyta) and *Fr. oceanica* (diatom) lost significant bioactivity (Table 2.7). The finding that bioactivity of *U. taeniata* is

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not tolerant of storage concurs with previous observations that bioactive compounds from Chlorophyta are notoriously unstable (Paul and Fenical, 1987). Despite diatoms being the largest microalgal population in marine ecosystems very little study of their metabolites as anti-infectives has been done (Shimizu, 1993), therefore no precedence on the chemical stability of their secondary metabolites was found.

The partial screening of algal extracts against tobacco mosaic virus found that only *F. gardneri* strongly inhibited infectivity of this virus (Table 2.5). *Tobamoviruses* and *Potexviruses* are not serologically related, differences between the surface moieties of these to viruses perhaps have led to this difference in efficacy of algal extracts (Brunt *et al.*, 1996). However, it is unusual that *F. gardneri* exerted an effect while the other active brown alga; *A. nana* did not, perhaps suggesting that the modes of action of these two brown algae are different. The assays were completed 2 months after the extraction of the algae, therefore compound degradation, especially in the case of extracts from the Chlorophyta and diatoms, may have contributed to the low overall effect. However, the same extract of *A. nana*, used two months later in the four-month assay, remained strongly antiviral against PVX (Table 2.7).

Several algal taxa identified as inhibitory against PVX also inhibited human viruses. For example, extracts of *Alaria nana*, *Codium fragile* and *Egregia menziesii* have been found to be antiviral against herpes simplex-1 (HSV-1) and/or Sindbis (Kim *et al.*, 1997; Hudson *et al.*, 1999a,b) and extracts of *Fucus vesiculosus* against human immunodeficiency virus (HIV) (Beress *et al.*, 1993). It is tempting to speculate that compounds with broad antiviral effect may occur in these taxa. However, differences in the nature of plant and animal viruses (i.e.: often membrane envelope) suggest that the common antiviral effect is the result of different algal constituents or modes of action.

As mentioned earlier, marine algae are not a monophyletic group, some belong to the Kingdom Plantae and others to the Kingdom Chromista. The algal sources of the most antiviral extracts in our study suggest a chemotaxonomic relationship based on this phylogenetic division. As Table 2.4 illustrates, 5 of the 6 most active algal extracts (*F. gardneri, A. nana, Ralfsia* sp., *E. menziesii* and *Fr. oceanica*) are from Heterokont taxa belonging to the Kingdom Chromista (Cavalier-Smith, 1998). The high representation of Heterokont species in the most antiviral extracts raises the possibility of inhibition being the result of common or related compounds. Members of the Heterokontophyta (brown algae) were identified in the early 1980's as a promising source of antiviral compounds (Rinehart, *et al.*, 1981); however, this trend is not reflected in studies using plant viruses (Galal *et al.*, 1999; Padmakumar and Ayyakkannu, 1997) or recent antiviral research on human viruses (reviewed in Schaeffer and Krylov, 2000; Hudson *et al.*, 1999a,b). Nonetheless, findings in this study suggest that this group of algae contains potent antiviral agents, and that further screening of extracts from Heterokont taxa could prove to be rewarding.

A high degree of variation was a considerable factor in a number of extracts, this variation appeared to originate from three possible sources: experimental, the local lesion host and algal substances. Sources of variation were reduced in the experimental design by combining control and test treatments on the same leaf and by standardizing plant age, nutrition, moisture, and light intensity between replicates. Some factors however cannot be regulated, one being the sensitivity of the assay. The local lesion assay relies on the hypersensitive response of *C. quinoa* which requires the presence of many viable virus particles for lesion development (Zaitlin and Palukaitis, 2000). Consequently the number of lesions on a leaf is dependent not only on the viability of the virus, but also on virus distribution over the leaf. surface and therefore is only an approximation of viable virus.

However for a large part, variation observed between replicates appears to be primarily an artifact of the host response. Although the local lesion assay serves the purposes of this study well, there is an inherent problem with variation (Matthews, 1991). This becomes apparent when the data are arranged in order of increasing viral inhibition; the standard error substantially diminishes as the potency of antiviral effect increases (Table 2.4). Kleczkowski (1949) suggested that such variation observed in local lesion assays is a function of an increase in the mean number of lesions; however in this study there was no such correlation. What may be contributing to this variation, which increases proportionately with percent infectivity, is a manifestation of the following. The hypersensitive response is induced by very specific host/pathogen interactions. However, the defense response (i.e.: lesion development or alternative event) is non-specific and involves metabolic changes specific to each host (Fritig *et al.*, 1990). Consequently, as the number of "infection events" (i.e.: lesions) increase relative to the control, it is possible that the non-specific nature (i.e.: variability) of the defense response becomes more apparent.

A further possible source of variation may come from the algal extracts themselves. As with any study involving natural populations: environmental, community, seasonal and individual factors can introduce variation between individual organisms (Duffy and Hay, 1990: Tuomi *et al.*, 1989). It is possible that compositional differences between specimens contributed to variation by influencing the concentration and/or presence of biologically active compounds. As Neushul (1990) described with reference to the Rhodophyta, variation is an inherent difficulty in working with algae. For example, abundance of the polysaccharide fucoidan within members of the brown algae varies dramatically with both the depth and season a specimen is collected (Percival, 1970). Furthermore, resident microorganisms may actually be the sources of active metabolites (He *et al.*, 2001; Shimizu, 1993). Therefore the presence or absence of resident bioactive microorganisms, epiphytes or endophytes associated with the algae could add to variation. An interesting observation related to this has to do with the high degree of variation found with specimens of *Mastocarpus* papillatus tetrasporophyte (Table 2.4). Microscopic evaluation of this species found highly variable amounts of unicellular *Acrosiphonia* sp. endophytes within its tissue. Perhaps, the high variability observed with the antiviral effect of extracts from *Mastocarpus papillatus* tetrasporophyte reflects differences in the abundance of Acrosiphonian or collaborative metabolites.

APPLICATION OF Fucus gardneri EXTRACT TO PVX INFECTED POTATO TISSUE CULTURE

Based on antiviral performance, extracts of F. gardneri appeared to be the most promising for chemical analysis; thus, this extract was further assayed for efficacy against systemic PVX infection. This bioassay, in contrast to LLAs, evaluated extracts for antiviral effect against established infections rather than the onset of infection. Treatments in this assay included three concentrations of crude extract and a semi-pure fraction, all verified as active in local lesion assays. However, after both 30 and 60 days of algal chemotherapy in tissue culture, all fractions failed to show antiviral effect (Table 2.8). This may indicate that active compounds are not effective against systemic infection; however, it could also simply reflect a problem with delivery of the active compounds to infected leaves. Previous work done with animal viruses has implicated biopolymers such as polyphenols, peptides and polysaccharides in the antiviral activity of algae (Schaeffer and Krylov, 2000). Is it possible that the size of the bioactive molecule(s) in F. gardneri caused problems with uptake by the roots? Compounds such as polysaccharides and polyphenols are capable of passing into either animal or plant root cells (Logeart et al., 1997; A.D.M. Glass, personal communication). However, it is possible that compounds responsible for the antiviral effect in LLAs may be degraded in tissue culture by extra-cellular hydrolases secreted by the roots (A.D.M. Glass personal communication). If this is the case, alternate protocols may help to avoid such problems by offering more direct delivery to the leaves, such as: application to the surface of infected leaves,

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injection into plant vascular tissue or to the media of virus infected plant protoplasts or animal cells. For example, J.B. Hudson (personal communication) found the crude extract of F. gardneri to have high antiviral activity against influenza A and herpes simplex-1, while remaining non-toxic to host cells. Providing that the same compounds affect plant and animal systems, the latter method may be the most effective method for testing the efficacy of compounds against established infection. With fewer steps to confound the assay, compounds could simply be absorbed from surrounding solution and viral effect monitored with ELISA.

In considering the toxicity observed in the most potent of the crude treatments $(10\mu g/\mu l)$ of tissue culture, it is important to bear in mind the high concentration and impure nature of the extract. Rather than being relevant to antiviral molecules, phytotoxic symptoms may be the result of elevated ionic concentration or toxicity of compounds not involved in antiviral activity. Although algae are clearly capable of toxicity, results of LLAs in this study and other antiviral studies (Kim *et al.*, 1997) found little toxic effect from active algal fractions. Thus once purified, active algal constituents may not elicit the phytotoxicity observed in the crude $(10\mu g/\mu l)$ tissue culture treatment.

As an aside, an algal polysaccharide has been found to reduce toxicity induced by AZT (azidothymidine) in HIV-infected cells. A synergy study looking at AZT and the algal constituent fucoidan, suggested that the therapeutic dosage of the more toxic AZT may be reduced when used in combination with algal fucoidan (Schaeffer and Krylov, 2000). Like AZT, ribavirin which is a common plant antiviral, is also a nucleoside analog. It is possible that fucoidan or other polysaccharides from *Fucus gardneri* could supply a similar synergistic effect in plant systems.

Conclusion

This study adds to the list of marine algae known to contain plant virus inhibitors and is the first to do so using potato virus X. Local lesion assays identified six of the thirty marine algae to contain compounds which strongly inhibit infectivity of this virus. Of these six, it is the first time that *Fucus* gardneri, Ralfsia sp. and Fragilaria oceanica have been reported to be a source of antiviral agents. Furthermore, when applied to the leaves of *C. quinoa* none of the 30 algal extracts produced symptoms of toxicity. The clustering of bioactivity in species from the Heterokontophyta is intriguing. Further screening of species belonging to this group and characterization of their active constituents presents an excellent opportunity for future work.

Performance of extracts from *F. gardneri* in both PVX and TMV screening, as well as dilution and compound stability assays clearly identified this species as the most promising for phytochemical analysis. The following chapter will cover the fractionation and isolation of semi-pure and pure active fractions of this alga. Limitations of this study were highlighted by the outcome of systemically infected *S. tuberosum*, these difficulties may be overcome with compound purification or use of alternate assays. Nonetheless the success of this screening emphasizes the potential of marine algae to provide abundant and renewable compounds for treatment of agricultural viruses. In the search to identify new and effective agrochemicals the oceans will without doubt hold many solutions.

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Chapter 3

Fractionation of Antiviral Compounds from Fucus gardneri

Introduction

Work to this point has identified several species of marine algae that exert a strong antiviral effect against PVX in local lesion assays (LLAs). *Fucus gardneri* was chosen over other species because of its potency in screening and dilution assays, local abundance and the stability of its biological activity. *Fucus gardneri* belongs to the order Fucales and is also known by the common names bladderwrack or bubble kelp. It can be found in abundance on rocks of the mid intertidal in a range stretching from central California to the Bering Sea, Chukchi Sea and Kamchatka (Figure 3.1). It forms a short and often dense canopy (<50cm) and is ecologically important as a source of food and protection from wave action and desiccation. Attached to rocks by a small discoid holdfast, a short stipe supports its flat, dichotomously branched thallus. At maturity, the tips of its branches are densely packed with gamete filled conceptacles. Living for between 2-5 years, individuals reproduce throughout the year (O'Clair and Lindstrom, 2000). Although the lifecycle involves alternation of generations, the haploid generation of *F. gardneri* is limited to the egg and sperm (Lee, 1999).



Figure 3.1: *Fucus gardneri* found growing densely on intertidal rocks of North Pacific coast. Source: King County Washington, 2001.
BACKGROUND

The goal of this phase of the project was to isolate and identify the compounds in *F. gardneri* that are responsible for the anti-PVX effects observed in chapter two. Based on a review of human virus research literature, the constituents of marine algae likely to have antiviral activity include: polyphenols, sulphated polysaccharides and peptides (Nakashima *et al.*, 1987; Baba *et al.*, 1988; Beress *et al.*, 1993; Boyd et al, 1997; Hudson *et al.*, 1999a,b). Few studies have evaluated the therapeutic potential of *F. gardneri*. There was no bioactivity in a screening against herpes simplex-1 and Sindbis viruses (Kim *et al.*, 1997); however, concentrations of aqueous *F. gardneri* extract as low as $20\mu g/ml$ did inhibit *Taq* DNA polymerase (Jin *et al.*, 1997). Nonetheless, there is no direct evidence of antiviral activity from *F. gardneri*.

Although I could not find specific studies on *F. gardneri*, previous work on the order Fucales has identified several hundred primary and secondary metabolites within the larger taxon (Valls and Piovetti, 1995). Phenolic compounds include phloroglucinol and other simple phenols like pyrogallol, catechol, resorcinol and hydroquinone (Waterman and Mole, 1994). The Fucales are also rich in diterpenes and compounds of mixed terpenoid shikimate biosynthetic origin (Valls and Piovetti, 1995). *Fucus vesiculosus* contains an abundance of phlorotannins and the polysaccharides alginic acid and fucoidan (Beress *et al.*, 1993). The biosynthetic origin of algal metabolites tends to be less complicated than in land plants. Below is a review of algal metabolites in *F. gardneri* considered to be primary candidates as sources of the observed anti-PVX activity.

Polysaccharides

Marine algae have a rich diversity of polysaccharides, which consist of both linear and branched chains (Lehninger et al., 1993) and serve a variety of roles by providing mechanical strength (Witvrouw and De Clerq, 1997), protection from desiccation, food storage (Percival, 1970) and perhaps even a mechanism for ion exchange (Eppley, 1958). Although variation occurs within each taxon, different forms of polysaccharides are characteristic of each of the major algal groups. Members of the Chlorophyta contain: starch, inulin, cellulose, D-mannans, β -D-xylans and mucilages (complex assemblages of carbohydrate, protein and sulphur). The Rhodophyta is characterized by: floridean starch, cellulose, β -D-(1 \rightarrow 4) mannans, β -D-xylans and sulphated galactans (i.e.: agars and carrageenans). The major polysaccharides of the Heterokontophyta include cellulose, alginate, fucoidan and laminarin or chrysolaminarin (Painter, 1983).

Cellulose

Cellulose is the only glycan that the Heterokontophyta shares with land plants. Although it is defined as a linear polymer of β -D-glucopyranose, algal cellulose often also contains mannose or xylose residues (Painter, 1983). As a principal component of cell walls, cellulose provides structure and strength to plants (Lehninger *et al.*, 1993). Due to its high molecular weight and strong associative properties, cellulose is only soluble under special conditions. Consequently, it is not likely that cellulose is involved in the observed bioactivity of methanolic extracts (Whistler and Smart, 1953).

Alginate

Alginate, the most well known polysaccharide of brown algae, is a collective term that refers to a family of linear $(1\rightarrow 4)$ -linked α -L-gulurono- β -D-mannuronans (Painter, 1983). These polyuronides are commercially important in food, dental and pharmaceutical applications (Percival, 1970). The key role of alginate in brown algae is structural, contributing as much as 40% of the dry weight. Alginate occurs as cationic salts (i.e.: Na⁺, Mg²⁺, Ca²⁺ & K⁺). Sodium salts of alginate are water-soluble, whereas alginates cross-linked with calcium or other

cations are completely insoluble (Van Den Hoek *et al.*, 1995). During extraction, I exploited this property by using Na_2CO_3 in an ion exchange reaction that transformed insoluble, mixed cation alginate into soluble sodium alginate (Haug, 1974).

Fucoidan

Fucoidans are sulphated L-fucans and although found in all brown algae, they are especially abundant in intertidal species such as *F. gardneri*. Fucoidans are found in intercellular tissues and extracellular exudates (Painter, 1983). They are extremely hygroscopic and are exuded by brown algae in the intertidal as protection against desiccation (Percival, 1970). As a consequence, the fucoidan content of this species depends on where individuals occur in the littoral zone (Lee, 1999). Although slightly soluble in aqueous solutions (w/v 3%), extraction of fucoidan under acidic conditions greatly increases yield (Percival and McDowell, 1967).

Laminarin

Found in all brown algae, laminarin, as the name suggests, was first isolated from the genus *Laminaria*. Laminarin is a food reserve and the abundance varies throughout the year, being plentiful in the autumn and winter and absent in the spring. Laminarins are $(1\rightarrow3)$ -linked β -D-glucopyranans containing 16 to 31 sugar residues and, unlike the previously described polysaccharides, are usually branched (Painter, 1983). The degree of branching as well as periodic $1\rightarrow6$ linkages and terminal mannitol residues introduce variation to different forms of laminarin (Lee, 1999). A high number of $1\rightarrow6$ linkages and/or branching points dramatically increase the solubility of these molecules, with many forms being soluble in cold water (Percival, 1970).

Phenolics

The term "phenolic" refers to a family of compounds containing an aromatic ring with one or more hydroxyl substituents. Such compounds are not directly involved in the primary metabolism and therefore are considered to be secondary metabolites (Mann, 1987). With the exception of one family of green algae, the Charophyceae, compounds from the shikimic acid pathway, such flavonoids and hydrolysable tannins, are absent in algae. Instead, metabolites such as phlorotannins, which are derived entirely from acetate, are found in abundance (Waterman and Mole, 1994).

Phlorotannins contribute as much as 10% to the dry weight (Ragan and Glombitza, 1986). Carbon-carbon and carbon-oxygen bonds link monomers of phloroglucinol (1,3,5-trihydroxybenzene) into polymers of various molecular weights (Haslam, 1998; McInnes *et al.*, 1985). Variations on the basic chemical structure include the addition of halogens, sulphates, hydroxyls and alkenyl side chains (Ragan, 1985).

Once considered to be waste products, such secondary metabolites are now recognized to mediate a host of ecological interactions. Although structurally different from polyphenols of land plants, phlorotannins are functionally similar (Tuomi *et al.*, 1989). One role for such compounds may be as nonspecific allelochemicals important in the regulation of endophytes, epiphytes, and bacteria and in herbivory (Lee, 1999).

Amino-based algal compounds

Polysaccharides and polyphenols are certainly the most widely reported classes of bioactive algal compounds; however, other algal substances have excellent therapeutic potential (Schaeffer and Krylov, 2000). One set of compounds that appear to be involved in the anti-PVX activity of *F. gardneri* are those which have amino groups. Amino functionalities occur widely in biological chemicals; however, this review will consider peptides, betaines and lectins.

Peptides

There is a growing awareness of the potential of peptides in therapeutic applications (Schaeffer and Krylov, 2000). Naturally occurring polypeptides range in length from two or three amino acids to more than a thousand. Large peptides are not easily extracted and may be degraded (Lehninger *et al.*, 1993), consequently I emphasize smaller peptides. These smaller peptides are considered to be most therapeutically relevant, because of uptake and physiological effects (Prasad, 1995). The small peptides, which have been isolated from algae are rich in arginine, asparagine or glutamine residues, although their function remains unknown. Nitrogen storage is a likely possibility (Higgins and Payne, 1982). An example of a very active antiviral peptide comes from the cyanobacterium, *Nostoc ellipsosporum*. This peptide, cyanovirin-N, has been selected for detailed study by the U.S. National Cancer Institute because of its potent action against HIV infection (Boyd *et al.*, 1997).

Lectins and Betaines

Lectins are proteins that specifically bind to carbohydrates, such as the glycans found on the envelope of some viruses. Lectins are found in both plants and animals, but their physiological role remains unclear, in part because lectins do not appear to have catalytic activity (Lehninger *et al.*, 1993). To date, no algal lectins have been implicated in antiviral activity. This may be more a consequence of research focus rather than a reflection of the bioactivity of algal lectins (Schaeffer and Krylov, 2000).

Betaines are methylated derivatives of amino and imino acids. Although not previously implicated in antiviral activity, they have been associated with crop stress reduction and are abundant in many algae (Blunden and Gordon, 1986). One, γ -aminobutyric acid betaine, found in *Fucus* sp. has recently been linked to the stimulation of plant defense response (Jenkins *et al.*, 1998) and is also well known for potent physiological effects in mammals (Liu *et al.*, 1993).

Methods and Materials

PRELIMINARY PHYTOCHEMISTRY

Local lesion assays

Unless specified, all LLAs discussed in this chapter were performed at a standard concentration of $10\mu g/\mu l$ as described in chapter 2. Most LLAs in this chapter were used as a tool for bioassay guided fractionation to direct isolation procedures. Results from these assays should be interpreted generally in this sense, not quantitatively.

Extraction of F. gardneri over a time course

The general polarity of bioactivity was determined by extracting F. gardneri with solvents of varying polarity. Each treatment began with 20g of dried plant material infused with 100ml of hexane or methanol (unless specified all organic solvents used were ACS grade, Fisher). Samples (20ml) were collected at 1 minute, $2\frac{1}{2}$ and 26 hours, dried *in vacuo* and assayed for bioactivity using LLAs.

Preparatory Thin-layer Chromatography

To determine the general polarity and position of bioactivity in the thinlayer chromatography (TLC) profile of the crude extract, two preparatory TLC plates (silica gel 60 F_{254} , EM Science) were developed using a chloroform:methanol solvent system (13:1) v/v. The plates were allowed to dry and elution profiles were determined using long and short wave UV lamps (Ultra-Violet Products inc.). Silica from individual UV visible bands was transferred to borosilicate tubes and extracted with 10ml methanol. This was repeated four times or until all color had eluted from the silica. Extracts of each band were dried *in vacuo* and assayed for bioactivity using LLAs.

Silica Gel Column Chromatography (Si-1)

This first column chromatography served to correlate further the elution profile with bioactivity. Using 25g of silica (70-230 mesh, Merck) in a 2x30cm column, 75mg of crude extract was fractionated with a chloroform:methanol:acetone (9:1:0 – 6:3:3) v/v/v gradient yielding 154 x 10ml fractions. Fractions were grouped according to TLC profile, dried *in vacuo* and assayed for bioactivity individually $(10\mu g/\mu l \text{ and } 20\mu g/\mu l)$ or in combination to test for synergy.

Autoclaving

As part of a tissue culture experiment, 100mg crude methanol extract was autoclaved for 15 minutes at a temperature of 120°C. Following autoclaving, sample was assayed for bioactivity using LLAs.

Methylation using diazomethane

Crude and semi-pure extracts were methylated in an effort to overcome difficulties in chemical isolation due to the extreme polarity of bioactive fractions. Methylated compounds are less polar and once isolated they can be demethylated using sodium hydroxide. This reaction was carried out using a diazomethylation kit (Aldrich Chemical Co.). The procedure relies on a reaction between diazald and an alcoholic alkali to produce diazomethane. Due to the toxicity of diazomethane and the risk of explosion, all preparations and the reaction were carried out in the fume hood.

Reagents (1ml diethyl ether, 1ml carbitol (monoethyl ether ethylene glycol) and 0.4g of diazald (Aldrich Chemical Co.)) were combined in the inner reaction vessel and the algal extract was dissolved in 3ml of diethyl ether and placed in the bottom of the outer vessel. The two vessels were assembled with an o-ring and a clamp. The base of the apparatus was placed in an ice bath to dissipate heat. Using a narrow gauge syringe, 37% potassium hydroxide (0.5 to

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1.5ml, BDH Chemical Co.) was carefully injected drop wise through the silicone rubber septum into the inner reaction vessel to begin the generation of diazomethane. The reaction rate was accelerated by gently shaking the apparatus every 10 minutes for the first 30 minutes and allowed to run overnight. The apparatus was disassembled and the diethyl ether was evaporated from the sample under a stream of nitrogen gas. Thin-layer chromatography (hexane:ethyl acetate 3:7) v/v and LLAs were used to follow changes to the extract.

Amberlite XAD-2 Column Chromatography (A-1)

Amberlite XAD-2 column chromatography (Beress *et al.*, 1993) was used to isolate the polyphenol portion of the crude extract. Amberlite XAD-2 is a polymeric divinylbenzene reverse-phase adsorbent with a high affinity for aromatic substances; thus is suitable for the isolation of alga phlorotannins (Gregson and Daly, 1982). A column (2x40cm) was prepared by adding presoaked Amberlite XAD-2 beads (Sigma) in water, followed by 2 volumes of methanol and water to wash the column. Crude extract (60mg) was charged to the column in a minimal volume of water and eluted using a water:acetonitrile gradient (100:0 – 50:50) v/v followed by a final wash with 3M ammonium hydroxide (Fisher). The final fraction (pH 11.4) was neutralized using dilute hydrochloric acid to a pH of 6.5. The resulting ammonium chloride was then separated from algal constituents by washing the fraction with acetonitrile. Fractions were grouped according to TLC profile and assayed for bioactivity using LLAs.

Polyamide Minicolumns

This method was designed to remove 99% of polyphenolic compounds from plant extracts, thus allowing for the evaluation of their possible role in bioactivity (Collins *et al.*, 1998). Holes were drilled in the base and lid of several plastic 1.5ml micro-centrifuge tubes (Dia Med) using a 20-gauge needle. A small amount of glass wool was packed at the base of each tube and an aqueous suspension of CC6 polyamide poured (Macherey-Nagel, Germany) to form a bed approximately 1cm deep. The micro-centrifuge tubes were then placed in the neck of 13 X 100mm glass test tubes and the polyamide in the minicolumn was rinsed with water and packed by centrifugation (GLC-1, Sorvall) for one minute (1000 x g) three times. One millilitre of aqueous extract (≤ 25 mg/ml) was applied to the column. Extracts were allowed to percolate and the column was rinsed with 1ml of distilled water and centrifuged for 1 minute as before and the eluate collected. This procedure was repeated with 1ml methanol:water (50:50) v/v and 1ml methanol. Fractions were dried *in vacuo* and assayed for bioactivity using LLAs.

Dialysis

An approximation of relative molecular weight was achieved using dialysis. Algal fractions were dissolved in a minimal volume of distilled water and dialyzed through a cellulose membrane (M.W. cut-off 12,000; Sigma). The surrounding solution of distilled water (4°C) was stirred constantly and replaced at intervals of 6, 21, 48 and 72 hours. These dialysate fractions were collected along with the final contents of the membrane, dried *in vacuo* and assayed for bioactivity using LLAs.

Sephadex LH-20 Column Chromatography (Se-1)

Sephadex LH-20 separates compounds primarily on the basis of molecular exclusion; resulting in further purification of active fractions based on differences in molecular weight. Sephadex beads (Pharmacia) were soaked for 4 days in methanol:chloroform (4:1) v/v, degassed *in vacuo* with constant stirring and poured to prepare a 2 X 40cm column, which was rinsed with two volumes of methanol:chloroform (4:1) v/v. The sample was a butanol fraction obtained from liquid partitioning. The fraction (49mg) was applied to the column in a

minimal volume of water-saturated butanol and eluted using a gradient of methanol:water (100:0 - 50:50) v/v.

Phytochemistry

Extraction and liquid/liquid Partitioning

The scale of extraction was increased using fresh material. Ten kilograms of *F. gardneri* was collected from Acadia Beach, Vancouver (49° 17' N, 123° 14' W) in December 2000. Material was washed, dried and ground, as previously described. Plant material was extracted at 45°C four times in 4L volumes of methanol. The supernatant was dried under vacuum at 35°C as previously described to produce a dried crude extract. The crude extract was suspended in 1200ml of distilled deionized water and partitioned against hexane (3800ml) in two 2L separatory funnels. Once separated, the water fraction was dried under vacuum to remove residual hexane and then partitioned against ethyl acetate (2400ml). Fractions were separated and residual ethyl acetate was removed from the water fraction *in vacuo*, which was then partitioned against butanol (1200ml). All four fractions were dried under vacuum and then assayed for bioactivity using LLAs.

Silica gel column chromatography (Si-2)

Based on results from previous silica gel columns, silica gel chromatography of the ethyl acetate fraction provided a further purification step. Using a silica to extract ratio of 1:25 the column (5x40cm) was filled with 325g of silica (600 mesh, Merck) and charged with the extract mixed with 62g silica (1:5) w/w. The column was eluted using a gradient solvent system with hexanes:acetone (80:20 – 0:100) v/v and then with acetone:methanol (100:0 – 0:100) v/v.

Sephadex LH-20 Column chromatography (Se-2)

Following silica chromatography, fractions were subjected to further purification using Sephadex LH-20 column chromatography (see *Se-1* above). Active fractions (3g) from silica gel chromatography (*Si-2*) were pooled (fractions 53-78) and applied in a minimal volume of methanol to a column (5x30cm) prepared with 30g Sephadex LH-20 beads. The column was eluted using an isocratic single solvent system beginning with 100% methanol (1500ml), followed by 100% acetone (650ml).

CHARACTERIZATION OF BIOACTIVITY

Spray reagents

Active fractions collected during this study were partly characterized using the following spray reagents (Stahl, 1969). Unless specified otherwise, all TLC was performed using aluminum silica gel 60 F_{254} plates (EM Science).

Ninhydrin (Stahl, 1969)

Ninhydrin reagent was prepared by dissolving 0.2g ninhydrin (Fisher) in 100ml ethanol. TLC plates were sprayed with the reagent and heated to 110°C. Positive reaction for amino acids, amines and aminosugars was indicated by the development of a pink/purple color.

Phenol sulphuric acid (Stahl, 1969)

Reagent was prepared by dissolving 3g phenol (Fisher) and 5ml concentrated sulphuric acid (Anachemia) in 95ml ethanol. TLC plates were developed, sprayed and heated at 100°C for 10-15 minutes. Bands containing sugars or glucosides were indicated by the development of a brown or yellow color.

Vanillin sulphuric acid (Stahl, 1969)

This reagent which is a general spray reagent identifying most organic compounds including higher alcohols, phenols, steroids and essential oils, was prepared by combining 1g vanillin (Sigma) with 100ml concentrated sulphuric acid (Fisher). Once plates were sprayed, color development was accelerated by heating at 120°C.

Folin-Ciocalteu reagent (Stahl, 1969)

This reagent, which is specific for phenolic compounds, was prepared by combining 10g sodium tungstate (BDH Chemical Co.) and 2.5g sodium molybdate (BDH Chemical Co.) in 70ml of water. To this, 5ml of 85% phosphoric acid (Fisher) and 10ml of 36% hydrochloric acid (Fisher) was added. The mixture was then refluxed for 10 hours. After cooling, 15g lithium sulphate (BDH Chemical Co.), 5ml water and 1 drop of bromine were added and the solution refluxed for a further 15 minutes. To develop TLC plates with this reagent, plates were first sprayed with 20% aqueous sodium carbonate (MCB Reagents) followed by spraying with a three times dilution of the stock solution. Bands containing phenolic compounds were indicated by the development of a grey color.

Dragendorff's reagent (Stahl, 1969)

Three variations of this reagent were prepared because of subtle differences in specificity. All were used to identify nitrogen-containing compounds containing quaternary nitrogen, including betaines. Heating was not required to produce a yellow to red color change indicating positive reactions.

 Reagent was prepared by dissolving 8g of bismuth nitrate (Baker and Adamson) in 20ml of 25% nitric acid (v/v) (Anachemia). The solution was mixed into a slurry of 20g potassium iodide (Sigma) with 1ml 6M

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hydrochloric acid (Fisher) and 5ml distilled water. The solution was diluted with water until a red-orange color developed and filtered.

- 2. Reagent was prepared by dissolving 0.85g bismuth nitrate (Baker and Adamson) into 50ml of 25% acetic acid (v/v) (Fisher). A second solution of 8g potassium iodide (Sigma) dissolved in 20ml water was added in equal proportions. The spray reagent was prepared by combining 1ml of the above stock solution with 2ml of acetic acid (Fisher) and 10ml of water.
- 3. Reagent was prepared by briefly boiling 25ml acetic acid, 2.6g bismuth carbonate (MCB Reagents) and 7g sodium iodide (JT Baker Chemical Co.). The solution was allowed to stand for 12 hours after which the abundant sodium acetate precipitate was removed by filtration. Twenty milliliters of the above solution were combined with 80ml ethyl acetate and 0.5ml water. The spray reagent was prepared by combining 10ml of the above stock solution with 100ml acetic acid and 240ml of ethyl acetate.

Lindt reagent (Ragan and Craigie, 1976)

Mix 1% (w/v) vanillin (Sigma) with a solution of 1 part 6M hydrochloric acid (Fisher) and 9 parts 95% ethanol. The presence of phloroglucinol is indicated by development of a red color.

Phloroglucinol reagent (Ragan and Craigie, 1976)

This reagent was used to identify the presence of phloroglucinol. It was made by preparing an aqueous solution of 0.1% (w/v) ferric chloride (Baker and Adamson) and 0.1% (w/v) potassium ferricyanide (JT Baker Chemical Co.).

Characterization of Fraction A *Dialysis*

Fraction A originates from Sephadex LH-20 column chromatography (Se-2) of extracts from *Fucus gardneri* (Figure 3.9). This fractionation led to the isolation of two active fractions, A and B, with fraction A being of lower molecular weight. Dialysis was used to obtain the crude approximation of the relative molecular weights of bioactive molecules in fractions A and B.

Amberlite IR-120 Column Chromatography (A-2)

This method (Blunden and Gordon, 1986) was used to isolate betaines and other quaternary ammonium or tertiary sulphonium compounds. Amberlite IR-120 ion exchange resin (Mallinckrodt) was pre-soaked in water overnight and 100mg of water-soluble crude extract was applied to the column. The column was eluted with 500ml of distilled water followed by 300ml of 3M ammonium hydroxide (pH 11.5, Fisher). The fraction was immediately neutralized with dilute hydrochloric acid (Fisher) to pH 6.5. Ammonium chloride was removed from this latter fraction by partitioning with butanol. Fractions were assayed for bioactivity using LLAs.

Acid-Urea Gel Electrophoresis

This method is designed to detect and isolate small cationic peptides. The protocol was provided by Dr. R.E.W. Hancock's research group (Department of Microbiology, University of British Columbia). The algal fraction was fraction A (fractions 6-9) from Sephadex LH-20 column (*Se-2*). The gels were poured and run using a Bio-Rad (U.S.A.) mini-gel system. The acrylamide solution was prepared by combining 3.2g urea (Baker and Adamson), 5.33ml acrylamide:bisacrylamide (30:0.8 w/w, Bio-Rad), 1.33ml water and 0.2ml 10% ammonium persulfate w/v (Bio-Rad). Gels were made by adding 30µl of TEMED (Bio-Rad) catalyst, mixing thoroughly and immediately pouring into

moulds. The loading comb was placed directly into the top of the running gel. Once set, gels were pre-electrophoresed to remove the ions with a running buffer of 5% acetic acid (Fisher). Run in reverse polarity at 150v, pre-electrophoresis continued until the current remained constant (1-2 hours). Poured gels were wrapped in Saran wrap and refrigerated overnight.

Samples were loaded in a sample solution, which was prepared as follows. A 10M urea (Baker and Adamson) solution was treated with Bio-Rad AG501-X8 mixed bed resin for the removal of anions from the solution. The sample solution was then prepared by combining 900µl of treated 10M urea with 50µl water and 50µl of acetic acid (final concentrations 9M-urea/5% acetic acid), to which a small amount of methyl green was added as a tracking dye. The extract was then dissolved (20mg/ml) in 5% acetic acid and mixed 2:1 v/v with the sample solution. 20µl aliquots were loaded into wells at the following concentrations: undiluted, 1/1, 1/10, 1/100 and 1/1000. The gel was run in reverse polarity at 150v for 1½ hours and at 15v overnight. The gel was stained using Coomassie blue (9 parts water:methanol (1:1) v/v, 1 part glacial acetic acid and 0.25g/100ml brilliant blue R-270(Sigma)) and destained using water:ethanol:glacial acetic acid (Fisher) (9:9:2) v/v.

SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Bio-Rad (U.S.A.) mini-gel system was used to detect and separate total protein in fraction A. The running gel was produced by combining the following ingredients in a Falcon tube: 1.3ml distilled water, 1.25ml 1.5M Tris-HCl (pH 8.8, BDH Chemical Co.), 25µl 20% SDS (BDH Chemical Co.), 2.5ml 30% acrylamide (Bio-Rad) and 25µl 10% ammonium persulphate w/v (Bio-Rad). Addition of 2.5µl of TEMED (Bio-Rad) catalyzed polymerization. The solution was then mixed and immediately poured between glass plates of the Bio-Rad mini-gel apparatus. The top of the running gel was immediately covered with a layer of isobutanol saturated with water to ensure a flat surface. The running gel was topped with a stacking gel for better separation. After allowing 40 minutes for the running gel to polymerize, the isobutanol was washed off with distilled water and the stacking gel was poured over top of the running gel. The stacking gel solution was prepared by combining 1.5ml distilled water, 625µl 0.5M Tris-HCl (pH 6.8), 12.5µl of 20% SDS, 325µl of 30% acrylamide and 12.5µl 10% ammonium persulphate w/v. As before, the polymerization was initiated by the addition of 2.5µl TEMED catalyst. Once poured, combs were placed at the top of the gel and solution was allowed to set for 1 hour. The algal extract used in this experiment was fraction A that had been further purified by boiling (described below- P-1). Proteins in fraction A (1.5mg) were denatured prior to electrophoresis by adding 40µl of loading buffer (15µl mercapto-ethanol (Sigma) and 400µl distilled water), sonicating and then heating at 90°C for 20 minutes. The entire 40µl sample was loaded into one lane and the gel was run using running buffer (2.3mM Tris pH 8.3, 19.2 mM glycine, 0.1% SDS) at 250v for 40 minutes. Once complete the gel was removed from glass plates and dyed in Coomassie blue stain (9 parts water:methanol (1:1) v/v, 1 part glacial acetic acid and 0.25g/100ml brilliant blue R-270) for one hour, destained for 2 hours in a solution of water:ethanol:glacial acetic acid (9:9:2) v/v/v and photographed using AlphaImage 1200 (Alpha Innotech Corporation).

Polyamide minicolumn

A portion of fraction A (fractions 14-20 @ 25mg/ml) was applied to polyamide columns and eluted with distilled, deionized water. Activity of the eluted solution was compared in LLAs to the original activity of fraction A.

Phloroglucinol experiment

To explore the possibility that phloroglucinol had an antiviral effect, a phloroglucinol standard (1,2,5-trihydroxybenzene, BDH Chemical Co.) was assayed for bioactivity against PVX. In addition, crude and semi-pure active fractions were chromatographed against a phloroglucinol standard in TLC. Plates were run using ether:ethanol:ethyl acetate:water (14:11:5.5:3) v/v/v/v and then sprayed with phloroglucinol specific spray reagents (Ragan and Craigie, 1976).

Polyamide Column chromatography (P-1)

found polyamide mini-columns Preliminary experiments that fractionated bioactivity in fraction A. As a result the process was scaled-up and applied to the entire fraction A (pooled with material from an earlier column, total-3.75g). In an attempt to solubilise fraction A for chromatography, the material was boiled in water for 2 seconds using a microwave, vortexed, immediately cooled and then centrifuged. The supernatant was verified to be antiviral using LLAs and then applied to the CC 6-AC polyamide (70g) (Macherey-Nagel, Germany) column. The column was washed with an isocratic solvent system beginning with water (100%), then water:methanol (50:50), methanol (100%) and finally acetone (100%). Fractions were clustered into 11 groups based on the TLC profile and assayed for bioactivity using LLAs.

Characterization of Fraction B Dialysis

Fraction B originates from Sephadex LH-20 column chromatography (Se-2) of extracts from *Fucus gardneri* (Figure 3.9). This fractionation lead to the isolation of two active fractions, A and B, with fraction B being of higher molecular weight. Dialysis was used to obtain a crude approximation of the relative molecular weights of fractions A and B.

Determination of sugars and related substances

This method (Dubois *et al.*, 1956) was used to confirm the presence of carbohydrates in fractions thought to contain polysaccharides. The reaction was carried out using test tubes with an internal diameter of 16-20mm to allow for good mixing while not allowing heat to dissipate too quickly. Agar (5.6mg; BDH Chemical Co.) was used as a positive control and distilled water was the negative control. Sample (6.1 mg fraction B) or controls were added to 2ml distilled water, 50μ l of phenol:water (80:20) v/v, and 5 ml of 95% sulphuric acid (Anachemia). Tubes were allowed to stand for 10 minutes, shaken and placed in a 25°C water bath for 20 minutes. A color change to yellow or brown indicated the presence of carbohydrates in the sample.

Ethanol Precipitation of Polysaccharides

Originally designed as a rapid method for dereplication of extracts containing sulphated polysaccharides in screening programs (Beutler *et al.*, 1993), this method was used to assess the role, if any, of sulphated polysaccharides in the bioactivity of *F. gardneri*. "The process of dereplication seeks to select a small subpopulation of hits identified in a primary screen that are most likely to contain active compounds with the desired characteristics." In this case ethanol precipitation removes polysaccharides which are often ubiquitous in marine extracts (Collins *et al.*, 1998). An aqueous solution of each crude methanolic or aqueous extract was prepared to a final concentration of 50mg/ml and an equal volume of ethanol was added. The solution was precipitated overnight at -20° C, centrifuged (1000 x g) and decanted. Both supernatant and precipitate were evaporated under vacuum and assayed for bioactivity using LLAs.

Acid hydrolysis

Acid hydrolysis of fractions was used to determine the monsaccharide constituents of the algal polysaccharides. Algal fractions (~200mg) were refluxed

in 5% hydrochloric acid (Fisher) overnight at 100°C. Hydrolysed algal fractions and mono- or polysaccharide standards were chromatographed on silica gel TLC plates using methylene chloride:methanol (8:3) v/v and developed with phenol sulphuric acid.

Schöniger's oxygen flask method

Sulphur analysis using this method was performed by Peter Borda of the Department of Chemistry, University of British Columbia using Schöniger's oxygen flask method (Hilp, 1988).

Transmission Electron Microscopy of PVX

Following up on work completed by Sano (1995), transmission electron microscopy (TEM) was used to evaluate the effects of fractions (crude extract, fraction A from *P-1*-frac.28, fraction B from *Se-2*-frac 1-2) from *F. gardneri* on PVX morphology and distribution. Preparation of treatment solutions was designed to reflect that of local lesion assays, therefore solutions of each extract ($10\mu I/\mu g$) were prepared using LLA control solution (aqueous 5% 2-methoxy ethanol). A 200µl aliquot of each solution was mixed with 10µl of PVX (1.15mg/ml), vortexed and allowed to incubate for 30 minutes. Electron microscopy grids (Ni 200) were prepared by applying 10µl of solution to the film surface for 1 minute. Solutions were then removed from grids using hardened filter paper (Whatman). Grids were then briefly treated with 10µl of 2% uranyl acetate (Canemco) in acetone and dried as before with hardened filter paper. Using a Zeiss 10C TEM (Germany), grids were viewed at magnifications between 31,500 and 50,000 x's.

Polysaccharide Isolation

The primary polysaccharides from F. gardneri were isolated according to modified methods of Mian and Percival (1973). Fresh plant material (1.5kg) was

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collected from Towers Beach, Vancouver (49° 16' N, 123° 15' W) in August 2001, washed, and ground fresh in liquid nitrogen using a mortar and pestle. Timing of this collection coincided with reported peak accumulation of carbohydrate reserves (Tuomi et al., 1989). The ground plant material was extracted twice with 6L of ethanol to reduce the amount of phenolic compounds that could otherwise contaminate polysaccharide fractions. Ethanol (1) was decanted and plant material allowed to dry. From this, 230g (dry weight) was extracted by constant stirring with 3L of 2% aqueous calcium chloride (BDH Chemical Co.) for four hours at room temperature. This yielded a laminarin and "fucans" fraction (2). Residual plant material was collected by filtration and further extracted in 3L of dilute hydrochloric acid (Fisher) at 70°C for 4 hours under constant stirring, during which time a pH of 2 was maintained by addition of further hydrochloric acid. This yielded a "fucans" fraction (3). A final extraction of the residual plant material was done with 3L 3% sodium carbonate (MCB Reagents) heated to 50°C and stirred constantly for 9 hours. This yielded an alginate and "fucans" fraction (4). Each of the three aqueous fractions (2-4) was reduced to a volume of 500ml in vacuo at 40°C. Crude polysaccharide fractions were further isolated by the addition of 1-1.5L of ethanol. Precipitated polysaccharide was removed by filtration and washed with additional ethanol.



Figure 3.2: Flow diagram of serial polysaccharide extraction of *Fucus gardneri* (modified from Main and Percival, 1973).

Each of the three crude polysaccharide fractions was fractionated using Sephadex G-100 (Sigma) column gel filtration. Sephadex G-100 (68g) was prepared for chromatography by boiling in distilled deionized water for 2 hours. The slurry was poured to build three columns (3x25cm). Precipitates (2. 14g, 3. 13g and 4. 15g) from each fraction were dissolved in 125ml of water and applied to a column. Columns were eluted isocratically using distilled, deionized water. Fractions were collected, dried *in vacuo*, pooled based on TLC profile and assayed for bioactivity in LLAs. Once isolated, polysaccharide fractions were assayed for bioactivity and active fractions compared with purchased standards (fucoidan, alginate and lamanarin) using acid hydrolysis. Hydrolysis was performed by refluxing samples and standards individually in 100ml 10% HCl v/v for 3 hours, simple sugar residues generated were compared on cellulose TLC plates (EM Science) using water-saturated butanol as the solvent system. For comparison, eight commercial polysaccharide standards (soluble starch (Nutritional Biochemical Co.), dextran sulphate (Sigma), dextran (Sigma), agar (BDH Chemical Co.), chitosan (Sigma), sodium alginate (Matheson Coleman and Bell), laminarin (Fluka) and fucoidan (Fluka)) were tested for antiviral effect in LLAs at $10\mu g/\mu l$. Alginate standard was tested over a concentration gradient $(33\mu g/\mu l - 0.01\mu g/\mu l)$ to evaluate the range of antiviral effect and any phytotoxicity.

Results

PRELIMINARY PHYTOCHEMISTRY

Extraction of dry plant material over a time course yielded levels of bioactivity dependent on the polarity of the extraction solvent (Table 3.1). Determination of bioactivity in solvent aliquots taken over the time course clearly showed that compounds responsible for the antiviral effect were preferentially soluble in methanol.

Table 3.1: Percent inhibition of PVX infectivity in LLAs by extracts from two different solvent extractions over a time course.

Infusion Time	Hexanes- % Inhibition	Methanol- % Inhibition
1 minute	26.0±19.0	100.0 ± 0
2.5 hours	-21.6±9.4	100.0 ± 0
26 hours	46.0±16.4	100.0±0

Positive values refer to viral inhibition, negative values refer to viral stimulation and \pm refers to the standard error of the mean.

In both cases bioactivity from preparatory-TLC was found exclusively at the origin of TLC plates. Table 3.2 is a summary of the bioactivity found on the plates with band 1 being the origin and band 8 the top of the elution profile (Figure 3.3). Similarly results from the initial silica column chromatography (*Si-1*) showed that the silica retained much of the bioactivity (Table 3.3). Antiviral activity that did elute from the column came near the end of the profile (fractions 151-154) and was weak (42-69%) regardless of concentration. Combinations of extracts tested did not show evidence of synergy between fractions. It should be noted that in these cases both preparatory–TLC and silica column chromatography used relatively non-polar solvent systems. % PVX Inhibition

profile.				
Band	1 (origin)	2	3	4
% PVX Inhibition	100±0	30.4±17.8	-73.7±26.6	-25.7±34.8
Band	5	6	7	8 (top)

Table 3.2: Percent bioactivity of compounds isolated from preparatory-TLC ь with h de nd . 1. . . 1.4 41 104

Positive values refer to viral inhibition, negative values refer to viral stimulation and \pm refers to the standard error of the mean.

13.5±13.2

 -54.8 ± 24.5

20.7±12.1

-49.7±16.6



Figure 3.3: Silica preparatory thin-layer chromatogram of crude Fucus gardneri extract. Numbers 1-8 represent the banding pattern tested for bioactivity (Table 3.2). Solvent system chloroform:methanol (13:1). Plate was developed using vanillin-sulphuric acid reagent.

Fractions	1-14	15-22	23	24-39	40-52	53-83
% PVX	-3.1	-3.6	-28.4	3.3	-16.0	-42.6
Inhibition	± 8.3	±17.4	±42.6	± 6.0	±7.0	± 55.3
Fractions	84-143	144-150	151	152	153	154
% PVX	-28.7	-44.9	68.6	46.0	42.7	69.1
Inhibition	±27.9	±19.0	±3.2	± 23.2	±12.4	±15.7

Table 3.3: Percent inhibition of PVX infectivity in LLAs by groups of fractions collected from silica column chromatography (*Si-1*).

Positive values refer to viral inhibition, negative values refer to viral stimulation and \pm refers to the standard error of the mean.

Thin-layer chromatography and LLAs of autoclaved crude extract found no changes with bioactivity remaining at 100% (Table 3.4, TLC not shown). Methylation of the crude extract yielded a dramatic change to both the biological activity and TLC profile (Table 3.4, Figure 3.4). Bioactivity of crude extract was reduced from 100% to less than zero. Controls showed that activity was soluble in diethyl ether (\blacksquare) and not simply precipitated during methylation (\Box).

Table 3.4: Percent inhibition of PVX infectivity of fractions collected before and after heat treatment and diazo-methylation of crude *F. gardneri* determined using LLAs.

Fractions	% PVX Inhibition
Autoclaved crude F. gardneri	100±0
Methylated crude F. gardneri	-46.2±47.2
Non-methylated crude F. gardneri	100±0
Crude in diethyl ether (■)	100 ± 0
Methylation precipitate ()	23.9±8.0

Positive values refer to viral inhibition, negative values refer to viral stimulation and \pm refers to the standard error of the mean.



Figure 3.4: Silica thin-layer chromatogram of crude (c) and methylated (m) *Fucus* gardneri extracts (Table 3.4). As this comparison demonstrates, methylation of the crude extract resulted in the generation of new compounds. Solvent system hexane:ethyl acetate (3:7). Vanillin-sulphuric acid reagent used to develop plate.

Amberlite XAD-2 column chromatography (A-1) of the crude extract yielded thirteen fractions. These were grouped according to TLC profile into four groups that correlated with changes in elution solvents (Table 3.5). Bioactivity as determined by LLAs was found primarily in the ammonium hydroxide fraction (pH 11).

Fractions	Elution Solvent	% PVX Inhibition	
1-4	Water	7.1±10.8	
5-8	Water:Acetonitrile (75:25)	52.6±18.0	
9-12	Water:Acetonitrile (50:50) 46.6±4.8		
13	3N Ammonium hydroxide	96.9±2.1	

Table 3.5: Percent inhibition of PVX infectivity in LLAs of fractions collected from Amberlite XAD-2 column chromatography (A-1).

Positive values refer to viral inhibition, negative values refer to viral stimulation and \pm refers to the standard error of the mean.

PRELIMINARY EVIDENCE SUGGESTING TWO ACTIVE COMPOUNDS

Dialysis was used to fractionate the crude extract early in the phytochemical analysis of F. gardneri. Over a period of 72 hours with regular changes in the solution, dialysis reached equilbrium. In the process, bioactive compounds were found both inside and outside the dialysis membrane. Sephadex LH-20 chromatography (Se-1) of an F. gardneri fraction yielded 54 fractions. These were clustered into five groups based on TLC profiles and when assayed for bioactivity yielded two distinct groups of fractions with antiviral activities of 73% (frac.4-7) and 95% (frac. 13-35) (Table 3.6).

Table 3.6: Percent inhibition of PVX infectivity of fractions collected from Sephadex LH-20 column chromatography (*Se-1*).

% PVX Inhibition
25.0±23.4
73.3±5.6
19.5±24.7
95.3±1.0
26.6±15.5

Positive values refer to viral inhibition, \pm refers to the standard error of the mean.

Phytochemistry

Extraction of 10kg of *F. gardneri* yielded 145g of crude extract that was verified by LLA to completely inhibit PVX infectivity. Liquid partitioning of this crude extract yielded hexane (26g), ethyl acetate (12.5g), butanol (64g) and water (42g) fractions that were also found to be all 100% antiviral. The ethyl acetate fraction was further fractionated using silica column chromatography (*Si-2*) yielding 100% bioactivity from fractions 53-78 (acetone:methanol 95:5 – 0:100) v/v (Table 3.7).

Table 3.7: Percent inhibition of PVX infectivity from silica column chromatography (*Si-2*) of the ethyl acetate fraction from liquid partitioning of crude *F. gardneri* extract.

Fractions	% PVX Inhibition	Fractions	% PVX Inhibition
1-4	47.6±7.3	32-38	33.8±14.0
5-8	2.8±4.1	39-52	-1.9 ± 42.6
9-12	-2.8 ± 46.3	53-59	100 ± 0
13-15	0.3 ± 23.6	60-63	100 ± 0
16-20	56.8±8.7	64-71	100 ± 0
21-25	11.5±36	72-73	100±0
26-31	26.2±13.6	74-78	100±0

Positive values refer to viral inhibition, negative values refer to viral stimulation and \pm refers to the standard error of the mean.

Sephadex LH-20 column chromatography (Se-2) of active fractions (53-78 - total 3g) obtained by silica chromatography (Si-2) yielded nine fractions averaging 240ml each (Table 3.8). Local lesion assays of these fractions revealed two distinct groups of bioactivity. Fractions 6-9 (2.4g) showed considerable bioactivity and fractions 1 and 2 (0.1g) were completely antiviral. These two groups of antiviral compounds will be referred to as Fraction A and Fraction B respectively (Table 3.8).

Fractions	Solvent	% PVX Inhibition	Reference
1	Methanol	100±0	EPACTION B
2	Methanol	99.9±0.1	I RACIION D
3	Methanol	81.4 ± 4.0	
4	Methanol	-3.1±13.3	
5	Methanol	65.5±4.3	
6-7	Methanol	87±4.4	EPACTION A
8-9	Acetone	100 ± 0	I RACIION A

Table 3.8: Percent inhibition of PVX infectivity in LLAs by fractions collected from Sephadex LH-20 column chromatography (*Se-2*) of silica column (*Si-2*) fractions (53-78).

Positive values refer to viral inhibition, negative values refer to viral stimulation and \pm refers to the standard error of the mean.

CHARACTERIZATION

Fraction A

In dialysis, bioactive compounds in fraction A passed through the cellulose membrane and into the surrounding distilled water. Thin-layer chromatography of fraction A yielded several bands that individually were reactive to Folin-Ciocalteu (phenols) and Ninhydrin (amino groups) and reacted slightly with phenol-sulphuric acid (carbohydrates) and Dragendorff's (alkaloids) reagents. At this point it was not clear which band was responsible for bioactivity of fraction A.

Betaines

Amberlite IR-120 chromatography (A-2) was used to address the possibility of algal betaines exerting an antiviral effect (Table 3.9). Chromatography of crude extract yielded water and ammonium hydroxide fractions. The latter fraction was partitioned with butanol, producing aqueous and butanol fractions. Spray reagents confirmed that the aqueous ammonia fraction was positive for both amino and quaternary nitrogen moieties.

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Table 3.9: Percent inhibition of PVX infectivity in LLAs by fractions collected	£
from Amberlite IR-120 column chromatography (A-2).	

Fractions	% PVX Inhibition
Water Fraction	98.1±2.0
Ammonia fraction – Aqueous*	89.0±14.9
Ammonia fraction - Butanol	16.5±16.3

*Positive reaction with dragendorff's reagent.

Positive values refer to viral inhibition, \pm refers to the standard error of the mean.

Peptides

Following up on the potential of amino-based compounds contributing to the antiviral effect, acid urea gel electrophoresis and SDS-PAGE were performed to look for anti-viral peptides. Acid urea gel electrophoresis, designed to isolate cationic peptides, did not reveal any such molecules (image of gel not included). Likewise SDS-PAGE, which screens for total protein, did not find discrete banding; however staining with Coomassie blue indicated the presence peptide-containing compounds in material from fraction A (Figure 3.5). The tailing portion of the electrophoresis profile was dyed, while the region below the leading edge fluoresced white under short-wave ultraviolet light.



Figure 3.5: Fluorescence image of electrophoresis of fraction A in SDS-PAGE. Left lane contains protein molecular weight standards. Right lane contains fraction A: Dark Coomassie blue stained tail (Coomassie is specific for basic and aromatic amino acids) (**C**); White UV fluorescence (**F**).

Phenolic compounds

To evaluate if antiviral activity was due to a phenolic compound, one fraction from group A was run through polyamide mini-columns. Activity was considerably reduced from 92.2% to 39% viral inhibition. Using appropriate spray reagents and TLC solvents, phloroglucinol, a common algal phenolic compound, was found to be present in active, crude and semi-pure fractions of *F. gardneri*. However, LLAs of phloroglucinol found no antiviral effect (4.5% stimulation).

Building on the results of polyamide mini-columns and in an attempt to resolve remaining difficulties with purity of active fractions, fraction A was applied to a large polyamide column (P-1). Polyamide chromatography of fraction A led to significant increases in purity in two ways. Firstly, by briefly

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boiling the desiccated fraction in water, compounds responsible for bioactivity became soluble (2.5g) while a considerable amount of material remained insoluble as a red precipitate. This aqueous solution was verified to be 100% antiviral and was run through the column yielding 28 fractions emerging in several clusters of activity (Table 3.10). Interestingly bioactivity eluted in three distinct groups. Thin-layer chromatography of eluted fractions found fraction 28 (99.3% inhibition) to be particularly pure. Thin-layer chromatography of this fraction strongly indicated both carbohydrate and amino compounds (ninhydrin positive) in the principal band.

Fractions	Elution Solvents Applied in Series	% PVX Inhibition
1-4	Water	52.9±20.7
5-6	Water	60.1 ± 20.0
7-14	Water	53.1±17.9
15	Water:Methanol (50:50)	59.8±18.8
16-19	Water:Methanol (50:50)	93.3±5.6
20-21	Methanol	58.6±23.0
22	Methanol	86.7±29.6
23-24	Methanol	100±0
25-26	Acetone	100 ± 0
27	Acetone	2.4±18.9
28	Acetone	99.3±0.6

Table 3.10: Percent inhibition of PVX infectivity in LLAs from fractions collected from Polyamide column chromatography (*P-1*) of fraction A of Sephedex LH-20 chromatography (*Se-2*).

Positive values refer to viral inhibition, \pm refers to the standard error of the mean.

Fraction B

Dialysis of fraction B from Sephadex LH-20 chromatography (Se-2) found that bioactivity remained within the cellulose membrane. Using TLC, this same fraction reacted positively with the phenol-sulphuric acid reagent (carbohydrates) but not Folin-Ciocalteu (phenols) or Dragendorff's (alkaloids) spray reagents. The presence of carbohydrates was confirmed using Dubois'

(1956) method, with both fraction B and agar (positive standard) solutions developing a distinct yellow to brown color.

Ethanol precipitation of the Sephadex LH-20 fraction B yielded a cream colored precipitate. Acid hydrolysis of this precipitate established it as crude polysaccharide (Figure 3.6). Thin-layer chromatography of hydrolysate, yielded four sugar residues one of which was glucose. Using Schöniger's oxygen flask method, Peter Borda of the Department of Chemistry (University of British Columbia) found sulphur to contribute 1.5% dry weight to the precipitate. Bioactivity of this precipitate was 100% while acid hydrolysed extract had no antiviral effect.



Figure 3.6: Thin-layer chromatogram of acid hydrolysed fraction B (**H**), crude fraction B (**C**) and glucose sugar standard (**G**). Arrows indicate glucose bands in standard and hydrolysed sample. Solvent system methylene chloride:methanol (8:3). TLC plate developed with phenol-sulphuric acid spray reagent specific for carbohydrates.

Transmission electron microscopy showed that PVX particles in the control appeared randomly arranged and interwoven, whereas particles exposed to the polysaccharide fraction (B) were almost exclusively aggregated in needle-like rafts (Figure 3.7). As a comparison, virus particles were also exposed to a strongly antiviral fraction from fraction A. Some aggregation of virus particles was observed; however, for the most part this treatment was comparable to

control. Images of virus treated with crude extract were obscured by undissolved material, thus are not included.



Figure 3.7: Transmission electron microscopy images of PVX particles after incubation in the following solutions: (i) negative control, (ii) polysaccharide treatment (fraction B), (iii & iv) alginate. Virus in negative control appears randomly arrange and non-linear, while virus treated with fraction B containing polysaccharide aggregated into needle-like rafts (iii) representative of most aggregates in alginate solution (iv) massive aggregation observed in several alginate preparations. Magnification x 31500.

Isolation of the specific bioactive polysaccharide (Mian and Percival, 1973) resulted in four crude fractions: **1.** ethanol extraction(3.55g), **2.** calcium chloride extraction(9.49g), **3.** hydrochloric acid extraction(3.96g) and **4.** sodium carbonate extraction(79.1g). All four crude fractions had strong antiviral effect in LLAs (Table 3.11). Crude polysaccharide fractions (2, 3 & 4) were fractionated using Sephadex G-100 chromatography yielding 8, 6 and 9 fractions respectively. Fractions from each Sephadex G-100 column were tested using LLAs. Only fractions from hydrochloric acid (3) and sodium carbonate (4) extractions were

found to completely inhibit PVX infectivity. These active, purified polysaccharides were hydrolysed and compared to simple sugars and standard laminarin, fucoidan and alginate using TLC. Results from chromatography of hydrolysed active fractions (Figure 3.8) and bioassays of commercial standards (Table 3.12) combine to implicate alginate as the source of bioactivity in fraction B. Alginate completely inhibited PVX infectivity at concentrations of $1\mu g/\mu l$, $10\mu g/\mu l$ and $33\mu g/\mu l$. Laminarin was the only other polysaccharide standard to show inhibition of PVX infectivity; however, chromatography of hydrolysates from the most antiviral polysaccharide fractions isolated did not show evidence of glucose (monomer of laminarin).

Table 3.11: Percent inhibition of PVX infectivity of crude polysaccharide fractions collected in a serial extraction of *F. gardneri*.

Fraction	% PVX Inhibition
Ethanol extraction	99.4±0.59
Calcium chloride extraction	98.6 ± 0.80
Hydrochloric acid extraction	84.1±14.76
Sodium carbonate extraction	85.7±2.08
	05.7 ± 2.00

Positive values refer to viral inhibition, \pm refers to the standard error of the mean.

Table 3.12: Percent inhibition of PVX infectivity of commercially prepared polysaccharides.

P = 1) = 1 = 1 = 1 = 1 = 1 = 1			
Polysaccharide	% PVX	Polysaccharide	% PVX
·	Inhibition		Inhibition
Soluble starch	-42.9±38.8	Chitosan	-63.2±44.7
Dextran sulphate	-35.4±47.2	Alginate	95.2±3.9
Dextran	-36.8 ± 30.5	Laminarin	56.6±25.1
Agar	-57.4±10.4	Fucoidan	-71.9±32.9

Positive values refer to inhibition of infectivity; Negative values refer to stimulation of infectivity. \pm refers to the standard error of the mean.



Figure 3.8: Thin-layer chromatography of hydrolysed alginate standard and active fractions from Sephadex G-100 chromatography. Chromatography profiles match. Using an alginate standard for comparison, the presence of alginate in active fractions (100%) of hydrochloric acid (4) and sodium carbonate (5) extractions was confirmed. Solvent system: water saturated butanol and 1 drop formic acid. TLC plate sprayed with phenol-sulphuric acid.
Discussion

PRELIMINARY CHARACTERIZATION

A comparison between extraction of the plant material over a 24-hour period with either hexane or methanol provided the first evidence that antiviral bioactive molecules from F. gardneri were polar (Table 3.1). The polar nature of the active material was confirmed using silica preparatory-TLC and column chromatography (*Si-1*). During these fractionations, elution of the antiviral agents was reduced by interaction with the polar silicate substrate. This effect was illustrated in the case of preparatory-TLC by bioactivity being found only at the origin of the plate, and in column chromatography by bioactivity eluting late from the column and with only marginal effect (Tables 3.2 and 3.3). These preliminary findings which found the bioactivity to be polar in nature, tended to rule out nonpolar compounds, like steroids or pigments, as being the source of antiviral activity.

Exposure of the crude extract to methylation, heat and alkali provided other valuable information. Effects of heat and alkali were chance observations made in association with other experiments. When autoclaved in preparation for tissue culture, samples of the crude extract remained 100% antiviral and no obvious changes in the extract's TLC profile were observed (Table 3.4, TLC not shown). Chromatography of extracts on Amberlite XAD-2 and IR-120 (A-1& A-2) provided interesting results because final elutions used 3M ammonium hydroxide. As with exposure to heat, in both cases bioactivity remained intact following exposure to pH 11 (Tables 3.5 and 3.9). It is difficult to make specific conclusions from these observations. The function of the active molecules appeared to be unchanged by heat and alkali; however, without further characterization it was not possible to rule out specific classes of molecules as possible sources of bioactivity. Diazomethylation helped me to deal with the highly polar nature of the bioactive compounds. By replacing hydroxyl groups with methyl groups, polar extracts were made more amenable to fractionation. Once purified, active compounds could be demethylated and compounds identified. Thin-layer chromatography and local lesion bioassays were used to gauge the effect of methylation on bioactivity and in both cases an obvious change in the properties of the crude extract was observed (Table 3.4 and Figure 3.4). Thin-layer chromatography profiles showed that there were twice as many bands in the methylated extracts than in the non-methylated material. This can be attributed to the generation of new compounds by methylation. Even more dramatic was the complete loss of bioactivity in methylated crude extracts. The knowledge that hydroxyl substituents are targeted during methylation hints at their importance in inhibition of PVX. This becomes important in understanding the mode of action of purification because of problems associated with demethylation.

DISCOVERY OF TWO ACTIVE FRACTIONS

In the development of a fractionation protocol, several forms of separation were attempted including dialysis and Sephadex LH-20 column chromatography. The first indication that bioactivity resided in more than one molecular source came during dialysis of the crude extract. Dialysis was performed until no further material passed through the membrane at 72 hours. Upon assaying the fractions, it was found that bioactivity was both retained and excluded by the membrane, suggesting that two different sized molecules were involved in the antiviral effect. Likewise, fractionation of extracts with Sephadex LH-20 column chromatography resulted in two active fractions of different relative molecular weight (Table 3.6). Upon realization that bioactivity was the result of more than one compound, the focus changed to scaling up extraction and isolation protocols.

Phytochemistry

Large-scale extraction and partitioning yielded hexane, ethyl acetate, butanol and water fractions, all of which were found to be completely antiviral. Finding bioactivity in non-polar fractions was a little surprising; and it seemed likely to be the result of over-extraction (recall Table 3.1). Nonetheless, the ethyl acetate fraction was chosen as a focus in hopes of targeting antiviral compounds of more moderate polarity. Silica column chromatography (*Si-2*) of the ethyl acetate fraction, as in the *Si-1* column, yielded bioactive compounds late in the elution profile (Table 3.7). Aside from being completely antiviral, these fractions all shared a deep red and highly polar compound. This red compound was associated with most, but not all, of the strongly active fractions produced throughout this study. Several attempts to isolate this compound using thin-layer and column chromatography unfortunately failed to separate it from similarly polar compounds.

The active fractions from silica chromatography (Si-2) were pooled and further fractionated using Sephadex LH-20 column chromatography (Se-2; Table 3.8). As before, two groups of active fractions eluted from the column: fraction A (low molecular weight) and fraction B (high molecular weight), from this point on these fractions were evaluated separately.

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Figure 3.9: Flow diagram of methods used leading to the isolation of fraction A and B from Sephadex LH-20 column chromatography (Se-2).

Characterization of Fraction A

With the separation of fractions A and B, the focus of the project changed to isolation and identification of their active constituents. Dialysis of fraction A established bioactivity to be water-soluble and small enough to pass through dialysis tubing (M.W. cut-off 12,000). Thin-layer chromatography identified phenolic, amino groups and possibly carbohydrates and quaternary nitrogen in this fraction; however, from this little else can be specified. Bioactivity could originate from any of the earlier mentioned classes of molecules; thus the role of each in bioactivity was addressed individually.

Polysaccharides

Using a protocol to dereplicate extracts for sulphated polysaccharides, fraction A was precipitated with ethanol. Upon the addition of ethanol to fraction A no precipitate resulted suggesting that polysaccharides were not present. The positive reaction for carbohydrates observed in TLC may have been in response to glucoside components of proteins or tannins.

Betaines

Following up on the suspicion that betaines may contribute to the anti-PVX effect of *F. gardneri*, isolation methodology from Blunden and Gordon (1986) was performed. To separate betaines from other algal metabolites, the extract was fractionated using Amberlite IR-120 column chromatography (*A-2*). According to Blunden and Gordon (1986), betaines would elute into the ammonia fraction. This "betaine" fraction, which was verified as containing both quaternary ammonium and amino groups, was found to reduce viral infectivity (Table 3.9). Interestingly, the ninhydrin reaction in this fraction was unique in that the color change was yellow, rather than purple suggesting an n-terminal proline. Although activity was found in the "betaine" fraction, chromatography achieved little purification and thus little confidence that betaines were in fact responsible for bioactivity.

Peptides and Lectins

With the finding that constituents of fraction A contained amino groups and the assistance of Dr. R.E.W. Hancock (Department of Microbiology, UBC), extracts of *F. gardneri* were evaluated for bioactive peptides. Natural peptides are known to have many therapeutic roles (Osusky *et al.* 2000; Zhang *et al.*, 2000), including the antiviral cyanovirin-N from *Nostoc ellipsosporum* (Boyd *et al.*, 1997). Acid-urea gel electrophoresis, which is specific for cationic peptides, was the first method used to isolate peptides. This method however did not yield any cationic peptides. Isolation of the total protein content of fraction A was then attempted using SDS-PAGE. Although this method was not successful in that discrete bands were not produced, some separation was achieved including one fraction that was positively stained by Coomassie blue (Figure 3.5). Coomassie blue is specific for basic and aromatic amino acids and therefore may indicate the presence of peptides. The molecular weight standards indicate that this region of the gel contains compounds with molecular weights larger than what would be expected for small peptides. Consequently it does not appear that such compounds are involved, however the diffuse and tailing nature of the stained region is consistent with the electrophoresis of glyco-proteins (I.E.P. Taylor personal communication). The contents of this band were sent for amino acid analysis; however, due to interruption of analytical services at both the University of British Columbia and the University of Victoria follow-up work on this fraction has not been completed.

Phenolics

Work focusing on phenolic compounds from F. gardneri has been the most conclusive of the characterization completed on fraction A. The role of phenolic compounds in bioactivity was confirmed by their selective removal using polyamide minicolumns. When run through polyamide, bioactivity from fraction A was dramatically reduced, indicating a role for polyphenols in antiviral activity. This finding was corroborated using Amberlite XAD-2 column chromatography (A-1), which allowed for the isolation of phenolic compounds (Table 3.5). In this case, bioactivity was concentrated in the ammonium fraction, which according to Beress et al. (1993), contains phenolics, particularly phlorotannins. Considering the abundance of phenolic compounds, especially those based on phloroglucinol, in F. gardneri these findings are not too surprising. Thin-layer chromatography with a phloroglucinol standard confirmed its presence in crude extracts; however, when assayed for bioactivity phloroglucinol (4.5% stimulation) itself was found to have no effect on PVX. It is likely that polymers of phloroglucinol are responsible for such bioactivity. Phenolic compounds from F. vesiculosus as well as other marine algae have been found inhibitory to HIV (Beress et al. 1993; Moen and Clark 1993), HSV-1 and Sindbis virus (Hudson et al., 1999a,b).

Full size polyamide column chromatography (P-1) was used in an attempt to isolate active phenolic constituents from fraction A. Preparation of fraction A for chromatography involved experimenting with brief boiling to solublize the material. While the bioactive components dissolved as a colorless solution, the deep red material that could not previously be separated (pg. 101) remained insoluble. The aqueous fraction was then run through polyamide and yielded three groups of bioactive fractions (Table 3.10). Although the purity of active fractions had improved, none consisted of a single compound and crystallization likewise did not yield active pure compounds. Of the three bioactive fractions, number 28 was the least contaminated and when tested the dominant band was strongly reactive to reagents for both amino groups and carbohydrates. This finding raises the possibility of a bioactive peptide; however, because of the problems with amino acid analysis further work on this fraction has remained unfinished.



Figure 3.10: Summary of methods and results from the characterization of Fraction A.

Fraction A summary

Although progress was made in the fractionation of fraction A, the chemical origins of bioactivity in fraction A remain undefined. Although a portion of bioactivity can be clearly attributed to phenolic compounds, possible contribution from peptides, glyco-proteins or betaines cannot be ruled out. Bioactivity resulting from a phenolic compound can likely be attributed to phlorotannins simply because other abundance and previous findings (Beress *et al.*, 1993). As the final polyamide column showed, there are at least two if not three active compounds in this fraction. It is this complexity, combined with what appears to be mixed biosynthetic origin (i.e.: glyco-protein) that explains the often non-specific and sometimes contradictory results observed.

FRACTION B

Based on the separation results from Sephadex chromatography, it was assumed, because of the elution order, that the molecular weight of fraction B was greater than fraction A. This was confirmed by dialysis. Unlike fraction A, the bioactivity of fraction B was retained by a semi-permeable membrane with a molecular weight cut-off of 12,000 daltons. An interesting correlation comes from Beress *et al.* (1993) who found that the most antiviral fraction of *F. vesiculosus* to be a polysaccharide fraction with a molecular weight between 10,000-20,000 daltons.

Further characterization of this fraction suggested that the antiviral constituent was a sulphated polysaccharide. Ethanol precipitation, designed for the dereplication of sulphated polysaccharides, yielded an antiviral precipitate. When sprayed with phenol sulphuric acid, thin-layer chromatography of this precipitate was positive for carbohydrate. Using agar as a positive standard, this result was corroborated by a colorimetric phenol-sulphuric acid reaction in solution. Evidence of the precipitate being a polysaccharide was provided by acid hydrolysis. When sprayed with phenol-sulphuric acid, chromatography of the acid hydrolysate clearly showed the generation of 4 sugars, one of which was glucose (Figure 3.6). Glucose is one of the most common constituents of algal polysaccharides, being found in cellulose and laminarin, and is liberated along with other simple sugars during hydrolysis of glycosidic bonds. Evidence that the polysaccharide may be sulphated came from elemental analysis, which reported fraction B to contain 1.5% sulphur by dry weight. Several previous antiviral studies have found that sulphated polysaccharides act against both plant and animal viruses (Gonzalez et al., 1987; Nakashima et al., 1987; Baba et al., 1988, Sano, 1995). Fucus gardneri contains sulphated polysaccharides, the most abundant being fucoidan and sulphated hexuronoxylofucans (Painter, 1983).

In work with tobacco mosaic virus, Sano (1995) found the sulphated polysaccharides chondroitin sulphate C and A strongly inhibited viral infectivity in LLAs. Electron microscopy of an *in vitro* preparation of virus and chondroitin sulphates showed an aggregation of the virus particles, thus reducing the functional concentration of the virus in solution. To determine if fraction B had a similar effect on PVX, virus treated with this polysaccharide fraction was observed using transmission electron microscopy. Using the incubation protocol of LLAs, the effect of fraction B on PVX was clearly similar to the findings of Sano (1995, 1999). While control treatment left viruses randomly arranged and supple, viruses exposed to fraction B were aggregated in needle-like rafts (Figure 3.7).

Confident that one of the antiviral agents in *F. gardneri* was a polysaccharide, I attempted to make a positive identification of the glycan involved by using an extraction method specific to polysaccharides. Ethanol precipitation of aqueous algal extracts does yield polysaccharide, but the material is invariably a combination of alginic acid, fucoidan and protein (Percival and McDowell, 1967). Instead, I chose a method designed for the sequential extraction of laminarin, fucans and alginic acid (Table 3.11). The first extract, calcium chloride, which contains crude laminarin and fucans, was found to inhibit PVX in LLAs (Mian and Percival, 1973). Likewise, both the aqueous HCl extract containing crude fucans and the sodium carbonate extract containing crude alginate and fucans were found to inhibit viral infectivity.

Following Sephadex G-100 chromatography of these extracts, active fractions were hydrolyzed and compared to polysaccharide standards. Comparison of TLC profiles and evaluation of bioactivity identified alginate as the antiviral (95%) polysaccharide (Table 3.12, Figure 3.8). Testing alginate over a range of concentrations $(33\mu g/\mu l - 0.01\mu g/\mu l)$, an antiviral effect was found to

occur down to a concentration of $1\mu g/\mu l$ with no indication of toxicity at any concentration. Referring back to work done on *F. vesiculosus*, a non-sulphated alginate fraction was also found to be antiviral against HIV (Beress *et al.*, 1993). Laminarin likely also contributed to the overall bioactivity observed in fraction B; however, bioactivity of laminarin standards was marginal and TLC of hydrolysates did not identify this polysaccharide in the most potent fractions.

Although earlier evidence in this study implicated fucoidan as a likely candidate for antiviral activity in fraction B, it appears that alginic acid and fucoidan were co-isolated in Sephadex LH-20 chromatography. Fucoidan standard was tested in LLAs and was found to actually stimulate PVX infectivity (Table 3.12). It is also important to note that anti-viral activity of polysaccharides is not necessarily widespread. Of the eight commercial polysaccharides tested for antiviral activity against PVX, six actually stimulated PVX infectivity and only alginate showed any considerable bioactivity (Table 3.12).

Furthermore, recent work by Sano (1999) has also identified commercial sodium alginates to be antiviral against tobacco mosaic virus in LLAs on *Nicotiana tabacum*. As with fraction B in this study and chondroitin sulphate A and C (Sano, 1995), alginate was found to cause aggregation of virus particles into needle-like precipitates. This more recent work helped to elucidate the antiviral mode of action of these polysaccharides. Not only does aggregation reduce the functional concentration of the virus, thus reducing the frequency of infection, but it may prevent penetration of viral nucleic acids into the cell by arresting decapsulation. In addition, anionic properties of the polysaccharides may also reduce infection by limiting interaction between cells and virus. It has been proposed that the infection process of tobacco mosaic virus in part relies on an attraction between cellular anionic polysaccharide could overwhelm such an

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interaction and thus suppress the rate of infection (Sano, 1999). Recalling the effect that methylation had on activity, a mode of action of this sort makes sense. For a large part, the anionic charge on polysaccharides comes from an abundance of hydroxyl groups. If these are methylated the polysaccharides would lose their anionic charge and consequently any mode of action dependent on it.

Despite the popularity of this charge mediated mode of action, there is strong evidence that it may not be the only antiviral effect that polysaccharides exert. In the past 8 years several studies have found polysaccharides to elicit plant defences and induce systemic acquired resistance (SAR) to crop pathogens (Klarzynski et al., 2000; Mercier et al., 2001; Kovalenko et al., 1993). Some algal polysaccharides, for example laminarin, are capable of mimicking natural elicitors, which upon recognition at the surface of the host cell stimulate the expression of plant defence genes (Mercier et al., 2001). The end result includes thicker cellwalls, production of phytoalexins, host hydrolyases and inhibitors of pathogen hydrolyases. These effects appear to be widespread, having been induced in assays using wheat, soyabean, alfalfa and tobacco. To date most studies have focused on testing resistance to the two most costly crop pathogens, fungi and viruses (Klarzynski et al., 2000; Mercier et al., 2001; Kovalenko et al., 1993). Although only laminarin and mannan sulphates, not alginate, have been implicated in systemic acquired resistance, the possibility of SAR as an additional mode of action is intriguing.



Figure 3.11: Summary of methods and results from the characterization of Fraction B.

Fraction B Summary

Isolation of alginate from *Fucus gardneri* and its identification as an antiviral agent against potato virus X is a first for plant virology. This is also the first screening of algae to look at the phytochemistry of antiviral activity in plant system. Initial indications that fucoidan is involved in antiviral activity have proven to be incorrect; however the finding that fucoidan stimulates infection is nonetheless interesting. In retrospect fraction B appears to have been an assemblage of molecules including alginate, laminarin and fucoidan. Transmission electron microscopy gave clear indications that alginate's antiviral mode of action relates to promoting aggregation of virus particles. Understanding the modes of action involved in alginate's antiviral effect, particularly possible modulation of plant defences, presents an excellent research opportunity.

SUMMARY AND CONCLUSIONS

In this research project, marine algae from coastal British Columbia were examined for compounds with bioactivity against the plant virus, potato virus X. Overall this project contributed to existing knowledge in this and related fields in several ways. This is the first study to evaluate New World algal species for compounds with potential for plant virus chemotherapy. Over thirty species were evaluated for bioactivity against potato virus X, extracts from six algal species (*Fucus gardneri, Alaria nana, Ralfsia* sp., *Codium fragile, Fragillaria oceanica, Egregia menziesii*) strongly inhibit PVX infectivity. The disproportionate representation of Heterokont species in these most antiviral extracts was unexpected and deserves further evaluation. Of these six species, it is the first time that *Fucus gardneri, Ralfsia* sp. and *Fragilaria oceanica* have been reported as sources of antiviral agents.

Phytochemical analysis has lead to isolation protocols and the identification of one of the primary antiviral constituents in *Fucus gardneri*. Crude extracts of *F. gardneri* undoubtedly were the most potent in preliminary antiviral screening and dilution experiments. Fractionation of the crude extract using partitioning, column chromatography and a variety of other separation protocols resulted in the isolation of two bioactive fractions. Antiviral activity of the lower molecular weight fraction (A) was the result of phenolic and maybe amino-based compounds that have yet to be positively identified. Phytochemical analysis of the higher molecular weight fraction (B) resulted in the isolation and identification of the polysaccharide alginate as the source of bioactivity. Alginate inhibited potato virus X infectivity dramatically, and preliminary electron microscopy studies of crude alginate extracts suggests the antiviral mode of action to be related to virus particle aggregation. To date most antiviral research relating to polysaccharides has focused on synthetic, animal or red algal polysaccharides (Witvrouw

and De Clercq, 1997). Follow-up work on alginate's mode(s) of action, particularly focusing on possible induction of plant defences may yield exciting results.

As was found in the screening of F. vesiculosus for anti-HIV activity (Beress *et al.*, 1993), bioassay guided fractionation of F. gardneri in the present study found bioactivity to be broadly distributed in a large number of fractions. Phytochemical analysis leaves little doubt that several antiviral compounds or a group of like compounds remain unidentified. The isolation and identification of these, as well bioactivity from the other top five extracts highlighted during screening, may be well worth the effort. Local lesion assays evaluating the antiviral activity of alginate and fractions of A showed no signs of phytotoxicity. Given the water solubility, natural abundance and apparent biologically benign nature of these antiviral agents, future work should include their evaluation in traditional chemotherapy protocols and determination of their effect against established infection.

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APPENDIX 1

Table A1.1: Percent inhibition of PVX infectivity of chalcones and crude extracts not discussed in thesis. Chalcones courtesy of Jeffery Harborne assayed at $20\mu g/ml$, extracts assayed at 10mg/ml.

Extract	Average % PVX	± Standard Error
	inhibition	
Wild Potato HAW1452 Stoloniferum	-60.7	±82.7
Wild Potato HJT349 Chacoense	34.0	±21.7
4'-methoxy 2'3-dihydroxy chalcone	-63.9	±55.5
2',4',3,4,-tetrahydroxy Chalcone (Butein)	-102.2	±34.1
4'-hydroxy 3,4,dimethoxy chalcone	-9.9	±31.8
6,3',4' trihydroxy aurone (Sulfuretin)	14.0	±41.1
2'-hydroxy 4',6',2,4 tetramethoxy chalcone	-81.2	±50.1
7-hydroxy-4'-methoxy chalcone	-100.6	±107.6
3',4' dimethoxy flavone	-24.8	±35.4
2'-hydroxy 3,4,5-trimethoxy dibenzoyl methane	-113.1	±65.1
2'-hydroxy 4',6',2,5-tetramethoxy chalcone	-68.8	±50.3
2'-hydroxy 3,4-dimethoxy chalcone	5.4	±14.7
2'4 diOH Chalcone	-60.5	±24.9
Acacetin	-13.0	±41.0
Devil's Club whole plant methanol extract	-1.5	±8.1
Feverfew surface dichloromethane extraction**	-22.9	±5.8
Feverfew whole plant methanol extract	7.3	±3.6
Lomatium dissecticum root methanol extract	98.4	±32.8
Lomatium dissecticum shoot methanol extract	14.4	±3.0
Lomatium geyeri root methanol extract	6.2	±10.4
Lomatium geyeri shoot methanol extract	0.4	±8.4
Lomatium macrocarpum root methanol extract	9.9	±17.5
Lomatium macrocarpum shoot methanol	-22.4	±35.4
extract		
Hypericum perforatum whole plant*	100.0	±0

*Extract provided by Andres Lopez **Extract provided by Kevin Usher.