DISSECTION OF TWO DISTINCT DEFENSE-RELATED RESPONSES TO AGAR OLIGOSACCHARIDES IN GRACILARIA CHILENsis (RHODOPHYTA) AND GRACILARIA CONFERTA (RHODOPHYTA)1

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The two agar-producing red algae, Gracilaria chilensis C. J. Bird, McLachlan & E. C. Oliveira and Gracilaria conferta (Schousboe ex Montagne) Montagne, responded with hydrogen peroxide (H2O2) release when agar oligosaccharides were added to the medium. In G. conferta, a transient release was observed, followed by a refractory state of 6 h. This response was sensitive to chemical inhibitors of NADPH oxidase, protein kinases, protein phosphatases, and calcium translocation in the cell, whereas it was insensitive to inhibitors of metalloenzymes. Transmission electron microscopic observations of the H2O2-dependent formation of cerium peroxide from cerium chloride indicated oxygen activation at the plasma membrane of G. conferta. A putative system, consisting of a receptor specific to agar oligosaccharides and a plasma membrane-located NADPH oxidase, appears to be responsible for the release of H2O2 in G. conferta. Subcellular examination of G. chilensis showed that the H2O2 release was located in the cell wall. It was sensitive to inhibitors of metalloenzymes and flavoenzymes, and no refractory state was observed. The release was correlated with accumulation of an aldehyde in the algal medium, suggesting that an agar oligosaccharide oxidase is present in the apoplast of G. chilensis. The presence of this enzyme could also be demonstrated by polyacrylamide eleetrophoresis under nondenaturing conditions and proven to be variable. Cultivation of G. chilensis at 16 to 17°C resulted in significantly stronger expression of agar oligosaccharide oxidase than cultivation at 12°C, which indicates that the enzyme is used under conditions that generally favor microbial agar macerating activity.

Key index words: Gracilaria; NADPH oxidase; oligoagar; oligosaccharide; oxidase

Abbreviations: DPI, diphenylene-iodonium; KCN, potassium cyanide; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NaN3, sodium azide; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid

Specific cell wall–derived oligosaccharides are known to regulate growth, development, and defense responses in higher plants (Darvill et al. 1992, Aldington and Fry 1993, John et al. 1997, Braam 1999, Vorwerk et al. 2004). Examples of recognition of oligosaccharides have also been reported for marine algae. Agar oligosaccharides released from the cell wall matrix of Gracilaria conferta (Schousboe ex Montagne) Montagne control the abundance of agar degrading bacteria at the surface of this alga (Weinberger and Friedlander 2000). Similarly, alginic oligosaccharides from the cell wall matrix of kelp sporophytes play a key role during the induction of resistance against algal endophytes and bacteria in the brown algae Laminaria digitata and Macrocystis pyrifera, respectively (Küpper et al. 2002).

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In plant and metazoan innate immunity systems, one of the earliest defense responses is the so-called oxidative burst, a rapid production of reactive oxygen species (ROS) such as \( \text{H}_2\text{O}_2 \) (Lamb and Dixon 1997, Wojtaszek 1997, Bolwell 1999, Laloi et al. 2004). Reactive oxygen species generated in the oxidative burst orchestrate the synthesis of defense-related proteins and phytoalexins (Apostol et al. 1987, Levine et al. 1994, Orozco-Cárdenas et al. 2001), and an increasing number of observations indicate that the oxidative burst after oligosaccharide recognition also mediates host–pathogen interactions in marine algae. Both \( G. \text{conferta} \) (Weinberger et al. 1999, 2001) and \( L. \text{digitata} \) (Küpper et al. 2001) react with an oxidative burst to the presence of agar or alginate oligosaccharides, respectively. Moreover, generation of \( \text{H}_2\text{O}_2 \) was shown to constitute an essential component of the defense system of the red alga \( C. \text{crispus} \) against the green algal endophyte \( A. \text{operculata} \) (Bourarab et al. 1999, Weinberger et al. 2002, 2005).

At least six different enzymatic sources of ROS have been identified so far in algal and plant defense systems. Apoplastic L-amino acid oxidase plays a role for the resistance of \( C. \text{crispus} \) toward \( A. \text{operculata} \) (Weinberger et al. 2002), whereas pH-dependent apoplastic peroxidase (Bolwell et al. 1998, Martinez et al. 1998), apoplastic carbohydrate oxidase (Custers et al. 2004), apoplastic copper amine oxidase (Rea et al. 2002), and apoplastic oxalate oxidase (Zhang et al. 1995) have been reported from spermatophytes. A membrane-located NADPH oxidase complex can be activated through defense elicitation in \( L. \text{digitata} \) (Küpper et al. 2001) and in spermatophytes (Auh and Murphy 1995, Doke and Miura 1995, Bolwell et al. 1998).

It is not known yet how plant NADPH oxidase is regulated, but increasing evidence (Blume et al. 2000, Navazio et al. 2002) indicates that a signal transduction cascade, including protein kinases, protein phosphatases, ion channels, changes in membrane potential, permeability, and ion fluxes, is involved (Wojtaszek 1997). This evidence is mainly based on pharmacological investigations conducted with known chemical inhibitors of mammalian enzymes. The same approach has also widely been used to distinguish between sources of ROS in the oxidative burst (Levine et al. 1994, Auh and Murphy 1995, Bolwell et al. 1998, Martinez et al. 1998). The specificity of chemical enzyme inhibitors is in most cases limited and dose dependent. For example, at relatively low concentrations diphenylene-iodonium (DPI) irreversibly binds to the flavonoid group of the NADPH oxidase complex (O’Donnell et al. 1993), whereas high concentrations of DPI also affect other enzymes potentially involved in the generation of ROS (Bolwell 1999).

In contrast with the ubiquitous response of higher plant cells upon challenge with either oligogalacturonans or oligoglucans, the capacity to recognize a specific oligosaccharide seems to be confined to some algal taxa. In six species of the Gracilariaceae that were tested, all responded with a respiratory burst when they were challenged with agar oligosaccharides (Weinberger et al. 1999). In contrast, such responses were not observed when other red algae, agarophytes or not, were examined. Sensitivity to agar oligosaccharides therefore seemed to be a ubiquitous but specific feature in the Gracilariaceae. However, the respective sources of ROS production were not clearly determined in these previous reports.

It was the purpose of the present study to identify the source of \( \text{H}_2\text{O}_2 \) that is activated in \( G. \text{conferta} \) after elicitation with agar oligosaccharides and to verify whether \( G. \text{chilensis} \) C. J. Bird, McLachlan & E. C. Oliveira responds in an analogous way. A system with similar pharmacological sensitivities as the receptor-activated NADPH oxidase in spermatophytes is shown to respond to agar oligosaccharides in \( G. \text{conferta} \). \( G. \text{chilensis} \), in contrast, contains an oxidase specific for agar oligosaccharides. Using for the first time cerium perhydroxide precipitation for the subcellular localization of ROS generation in seaweeds, we show that \( \text{H}_2\text{O}_2 \) generation is apoplastic in \( G. \text{chilensis} \) but plasma membrane located in \( G. \text{conferta} \).

**MATERIALS AND METHODS**

- **Plant material and cultivation procedures.** \( G. \text{chilensis} \) C. J. Bird, McLachlan & E. C. Oliveira originated from Caldera, IIIrd region of Chile. The isolates used were CR14, CR16, and CS7. Further isolates from Caldera, as well as from La Herradura and Praia Changas (IVth region), were also used for PAGE, whereas strain CUA was collected at Curaco (Xth region). \( G. \text{chilensis} \) and \( G. \text{conferta} \) (Schousboe ex Montagne) Montagne from Israel were cultivated in aerated tanks. The water temperature was maintained with heat exchangers at 25° C in the case of \( G. \text{conferta} \) and at 12 to 17° C in the case of \( G. \text{chilensis} \). Day length was 12 h, and the photon flux density was 45 \( \mu \text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \). During light exposure, a halogen lamp provided G. conferta every hour for 30 min with additional 80 \( \mu \text{M} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \). The water was exchanged daily, and nitrate and phosphate were supplied by weekly pulse feeding. Short-time incubations for elicitations and physiological assays were conducted in Petri dishes or Erlenmeyer flasks on shakers, using autoclaved seawater as medium. Algal fresh weight density was generally 50 mg \( \cdot \text{mL}^{-1} \), and the temperature during incubation was 13° C for \( G. \text{chilensis} \) and room temperature for \( G. \text{conferta} \).

- **Chemicals and oligosaccharides.** All chemicals and solvents were from Sigma (St. Quentin, France) and Merck (Darmstadt, Germany). Pharmacological agents were added from stock solutions prepared with DMSO or with water in the cases of potassium cyanide (KCn), sodium azide (NaN3), and 4-aminoypyridine. For the production of agar oligosaccharides, f-agarase from Zobellia galactanivorans was produced and used to degrade agarose (lot no. GC539223, Eurogentec, Seraing, Belgium) as described earlier (Allouch et al. 2005). Size calibrated neogalactobiose and neogalactotetraose and a fraction containing agar oligosaccharides consisting of more than four monosaccharide residues were prepared by gel filtration of the hydrolysate on a BioGel P-2 column (Bio-Rad, Hercules, CA, USA). Reduced agar oligosaccharides were prepared by incubation with sodium borohydride as described in Weinberger et al. (2001). All saccharides were added from stock solutions containing 10 mg \( \cdot \text{mL}^{-1} \) that were
Two Responses to Agar Oligosaccharides

H₂O₂ quantification. Hydrogen peroxide in the algal medium was quantified as lumino-dependent luminescence, using a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). A sample volume of 300 µL was mixed with 100 µL of a 0.3 mM luminal solution and 50 µL of phosphate buffer (10 mM, pH 7.8) containing 200 U mL⁻¹ horseradish peroxidase (Boehringer, Mannheim, Germany), and the luminescence was quantified immediately. In experiments requiring application of enzyme inhibitors, 250 µL of a 15 mM potassium ferricyanate solution was injected into the samples as catalyst instead of peroxidase (Bolwell et al. 1998), and seawater buffered with 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (pH 7.6, 50 mM) was used as medium. Under such conditions, only KCN interfered with the assay and increased its sensitivity in a dose-dependent manner by up to 16.5 times. Results obtained with KCN were corrected accordingly. In dose-response experiments, H₂O₂ concentrations were quantified 30 min after addition of agar oligosaccharides to the medium of G. chilensis. In analogous experiments with G. conferta, four H₂O₂ measurements were conducted 2.5, 3.5, and 4 min after exposure of G. conferta to agar oligosaccharides, and the mean of the four data obtained was calculated. Data obtained in dose-response experiments were fitted by iterative adaptation to logistic or Michaelis-Menten functions. In addition to the treatments (a), three different controls were run in all experiments to allow for compensation of H₂O₂ scavenging and H₂O₂ re-release due to uncontrolled effects. These were (b) medium treatment, (c) medium + alga, and (d) medium only. The net-release of H₂O₂ was then calculated according to the formula: 

\[ \text{Net-release} = \frac{\text{Net-release}_{\text{a} + b} - (c - d)}{\text{Algal density}} \]

The determination of H₂O₂ scavenging potentials, approximately 15 µM H₂O₂ were added to the medium of Gracilaria and the decrease of H₂O₂ concentrations in the medium was followed during 40 min.

Subcellular localization of H₂O₂. The cytochemical method for localization of H₂O₂ was based on the generation of insoluble cerium peroxides and adapted from Bestwick et al. (1997). After inoculation with or without agar oligosaccharides for various time periods, Gracilaria pieces (2–4 mm) were excised and fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h. Smaller Gracilaria pieces (0.5–1 mm) were then excised and fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 0.25 M sucrose. The original sucrose concentration was reduced by 25% in each successive step. After two incubation intervals. After completion of the incubation, 0.2 mL acetate buffer (0.2 M, pH 4.0) and 0.2 mL of a 0.5% aqueous solution of 3-methyl-2-benzothiazolinone hydrazone were added to all tubes, and they were incubated for 3 min at 100 °C. After cooling to room temperature, 1.25 mL of a freshly prepared solution of iron (III) chloride (0.2%) was added and the reacting tetrazopentamethine cyanine dye was quantified at 670 nm, using galacturonic acid as standard.

RESULTS

Kinetics of H₂O₂ release in response to agar oligosaccharides differ in Gracilaria chilensis and Gracilaria conferta. Exposure to agar oligosaccharides resulted in an immediate release of H₂O₂ in both G. chilensis and G. conferta yet with markedly different kinetics (Fig. 1). In G. chilensis, maximal H₂O₂ concentrations were typically observed from 20 to 30 min after the addition of 300 µM agar oligosaccharides to the incubation medium, and they remained stable for more than 30 min. Transfer of G. chilensis into a fresh me-

![Graph showing kinetics of H₂O₂ release](image)

**Fig. 1.** Kinetics of H₂O₂ concentrations in the medium of Gracilaria chilensis (○) and G. conferta (●) during two cycles of exposure to agar oligosaccharides (black arrows) upon replacement of the medium with fresh medium (white arrow).
Table 1. Effects of pharmacological agents on H$_2$O$_2$ release by *Gracilaria chilensis* and *G. conferta* after challenge with agar oligosaccharides.

| Reagent          | Effect       | EC$_{50}$ or dose tested (µM) | Effect       | EC$_{50}$ or dose tested (µM) |
|------------------|--------------|------------------------------|--------------|------------------------------|
| Chlorpromazine   | Full inhibition | 20 (10.7–37.5) | Full inhibition | 18 (7.8–41.2) |
| Quinacrine       | Full inhibition | 41.2 (17.7–95.9) | Full inhibition | 22.8 (13.6–38.3) |
| DPI              | No           | 100                         | No           | 100                         |
| SHAM             | Full inhibition | 14.2 (5.2–38.3) | Full inhibition | 0.15 (0.079–0.285) |
| KCN              | Full inhibition | 959 (796–1155) | No           | 100                         |
| NaN$_3$          | Full inhibition | 758 (310–1854) | Full inhibition | 60–200                      |
| Staurosporine    | No           | 100                         | Full inhibition | 73.7 (53.7–132.0) |
| Cantharidine     | No           | 100                         | Full inhibition | 1.5 (0.2–10.2) |
| Bepridil         | No           | 100                         | Full inhibition | 46.2 (31.9–66.9) |
| Verapamil        | No           | 100                         | Full inhibition | 100                         |
| A23187           | No           | 100                         | Full inhibition | 999 (519–1922) |
| 4-Aminopyridine  | No           | 1000                        | Full inhibition | 999 (519–1922) |

The incubation times after exposure to agar oligosaccharides were 45 min and 4 min for *G. chilensis* and *G. conferta*, respectively. Either the tested doses or the necessary doses for half maximal (EC$_{50}$) inhibition (with 95% confidence intervals in brackets) are given.

dium followed by addition of agar oligosaccharides immediately resulted in a renewed release of H$_2$O$_2$. With *G. conferta*, maximal concentrations of H$_2$O$_2$ in the medium were reached less than 10 min after the addition of 30 µM agar oligosaccharides. In this alga, however, a desensitizing effect was apparent and resulted in a single oxidative burst after the first elicitation. Subsequent challenge with 30 µM agar oligosaccharides did not trigger the same response.

In *G. chilensis* and in *G. conferta*, no H$_2$O$_2$ accumulation was detected after exposure to agar oligosaccharides when 1000 U·mL$^{-1}$ catalase was added to the medium (data not shown). The responses of the two species were differently affected by the pH of the medium. In *G. chilensis*, a maximal release was observed at pH 7.8–8.5, whereas *G. conferta* maximally responded at pH 7.0 (data not shown).

**Inhibitor studies.** Of 18 pharmacological agents that were tested, only chlorpromazine, quinacrine, salicylhydroxamic acid (SHAM), KCN, and NaN$_3$ inhibited the release of H$_2$O$_2$ in *G. chilensis* (Table 1). In contrast, the response of *G. conferta* was uninhibited by SHAM, KCN, and NaN$_3$ but sensitive to a wide range of other compounds (Table 1): The oxidative burst was inhibited by chlorpromazine and quinacrine and was particularly sensitive to DPI. Staurosporine, a general inhibitor of protein kinases, reduced the H$_2$O$_2$ release by 50% at 73.7 µM, whereas the protein phosphatase inhibitor cantharidine approxi-
mately doubled it when it was present at similar concentrations. Bepridil and verapamil, as well as other blockers of calcium channels (flunarizine and diltiazem, data not shown), generally inhibited the release of H₂O₂. A certain inhibition was also observed with ophiobolin A, an inhibitor of the calmodulin system, and the calcium ionophore A23187 increased the response. A full inhibition was observed when relatively high concentrations of the potassium channel inhibitor 4-aminopyridine were applied.

Presence of NaN₃ or KCN in the medium of *G. conferta* increased the release of H₂O₂ after challenge with agar oligosaccharide (Table 1), and this increase was correlated with a decrease in the algal capacity to scavenge H₂O₂ (Fig. 2). Application of 100 μM NaN₃ still inhibited most H₂O₂ scavenging in *G. conferta* and thus allowed a more sensitive detection of H₂O₂ release. It was in this way possible to demonstrate that the recovery of *G. conferta* from the refractory state began 2.5 h after exposure to agar oligosaccharide, because *G. conferta* began at that time with a new release of H₂O₂ when agar oligosaccharide was present in its medium (Fig. 3). As with the first response (Table 1), this new H₂O₂ release was sensitive to 10 μM DPI and uninhib-
Table 2. Kinetic constants for H2O2 release by Gracilaria chilensis after exposure to agar oligosaccharides with three different degrees of neoagarobiose polymerization.

| Degree of neoagarobiose polymerization | Vmax (nmol g⁻¹ min⁻¹) | 95% confidence interval | Kₘ (µM) | 95% confidence interval |
|---------------------------------------|------------------------|-------------------------|---------|-------------------------|
| 1                                     | No response            | —                       | —       | —                       |
| 2                                     | 2.3                    | 2.0–2.6                 | 138.8   | 102.3–175.4             |
| >2                                    | 1.2                    | 1.0–1.3                 | 33.1    | 14.8–51.4               |

The maximal responses (Vmax) and the necessary doses for induction of half maximal responses (Kₘ) are given with their 95% confidence intervals.

...continued. The capability of G. conferta to respond with an oxidative burst to agar oligosaccharides was nearly fully restored after 5 h (Fig. 3).

Sites of H2O2 production in response to agar oligosaccharides. When G. chilensis was incubated in the presence of both cerium chloride and agar oligosaccharides, several layers of electron-dense material appeared in the cell wall (Fig. 4, A and B), accumulating at the outer cell wall. This cerium perhydroxide precipitate became visible after 30 to 70 min of exposure to the agar oligosaccharides. Cerium perhydroxide did not precipitate when no agar oligosaccharides had been added to the medium (Fig. 4C). In G. conferta, electron-dense material was visibly accumulated at the plasma membrane as early as 1 min after agar oligosaccharides had been added to the medium (Fig. 4, D and E). After 7 min, dense layers of cerium perhydroxide deposits were observed at the plasma membrane, not only in the epidermal cells but also in the second layer of cortical cells (Fig. 4F). Precipitates of cerium perhydroxide were not observed in the controls (Fig. 4G).

Gracilaria chilensis displays an agar oligosaccharide oxidase activity. No release of H2O2 by G. chilensis was observed during incubation with oligosaccharides prepared from carrageenans, fucan, alginates, ulvan, pectins, chitins, or cellulose (data not shown). Neoagarobiose did not induce H2O2 release in G. chilensis, even when it was present at concentrations as high as 1 mM. The response to the presence of agar oligosaccharides larger than neoagarobiose was dose dependent, with a higher maximal response and consequently a higher Kₘ toward neoagarotetraose than toward larger agar oligosaccharides (Table 2). The highest responses were observed when agar oligosaccharides were present in the medium at concentrations of 300 µM or higher.

After reduction with sodium borohydrite, agar oligosaccharides did not induce release of H2O2 in G. chilensis at concentrations as high as 1 mM. Moreover, the application of 1 mM of reduced agar oligosaccharides in combination with 130 µM of nonreduced agar oligosaccharides resulted in a slower release of H2O2 compared with the application of 130 µM agar oligosaccharides alone (Fig. 5).

Development of native PAGE gels loaded with G. chilensis protein extracts in the presence of phenazine methosulfate and MTT revealed five additional bands (1–5, Fig. 6) when agar oligosaccharides were present in the incubation buffer. No additional bands developed in G. conferta in the presence of agar oligosaccharides (data not shown). None of the bands observed with G. chilensis was detected when the development of PAGE gels was conducted in the presence of quinacrine, chlorpromazine, or SHAM or when agar oligosaccharides were replaced with D-galactose. Bands 1 to 3 were detected in extracts from all 15 G. chilensis individuals that were examined, whereas bands 4 and 5 were only detected in extracts from strain CUA. The staining intensity of bands 1 to 3 did not differ significantly between the 15 different strains due to a generally high variability over time in all strains (data not shown). This variability resulted in part from variations in the temperature regime during cultivation: Incubation of G. chilensis for 6 or more weeks at 16°C resulted in a staining intensity of lanes 1 to 5 that was nearly seven times as high as after incubation for 10 weeks at 12°C (Fig. 7).

An enrichment in carbonylic and aldehydic compounds, but not in uronic acids, was observed when G. chilensis was incubated in the presence of agar oligosaccharides (Fig. 8). The molar ratios of carbonyl compounds and aldehydes to the agar oligosaccharides initially applied were approximately 0.85:1. In contrast, only about 0.1 mol of carbonyl compounds and aldehydes were generated per mol of agar oligosaccharides previously reduced with sodium borohydrite.

![Graph showing release of H2O2 by Gracilaria chilensis after exposure to agar oligosaccharides](image-url)

**Fig. 5.** Release of H2O2 by Gracilaria chilensis after exposure to 130 µM agar oligosaccharides (●), 1 mM reduced agar oligosaccharides (×), and 130 µM agar oligosaccharides in combination with 1 mM reduced agar oligosaccharides (○). Bars indicate ± SD, n = 5.
Aldehydic compounds were also generated when agar oligosaccharide was added to crude protein extracts of *G. chilensis*. Production at a constant rate was typically observed during 2 h after the addition, whereas longer incubation periods usually resulted in reduced activity (Fig. 9A). A decrease in the aldehyde concentration after an initial increase was observed with some protein extracts, indicating that they contained components that decomposed the reaction product. Extracts that showed such interference were systematically excluded from the evaluation. Protein extracts that generated aldehyde at a relatively high rate generally also resulted in a strong agar oligosaccharide oxidase staining in native PAGE (Fig. 9B).

**DISCUSSION**

We show that the two species of *Gracilaria* under investigation responded with a release of H$_2$O$_2$ that was sensitive to catalase when agar oligosaccharides were applied to their culture medium. However, all the evidence we gathered indicates that two distinct cellular mechanisms are responsible for the release of extracellular H$_2$O$_2$, leading to different consequences in the defensive capabilities of these species.

*Agar oligosaccharides are perceived as a signal by Gracilaria conferta.* In *G. conferta*, a transient peak of H$_2$O$_2$ in the medium was observed for some minutes after the application of agar oligosaccharide, and this response could not be reinduced by supplying fresh medium and new agar oligosaccharides. The effect could be reverted only several hours after the first oxidative burst and the capacity to respond regenerated successively, indicating that the algal system of H$_2$O$_2$ production was in a refractory state as it is typically the case after recognition of chemical signals that activate phosphorylation events (Felix et al. 1993, Navazio et al. 2002). This view is further supported by the fact that the protein kinase inhibitor staurosporine inhibited the H$_2$O$_2$ release by *G. conferta*, whereas the protein phosphatase inhibitor cantharidine increased it. A complete reinduction of
The release of ROS by *G. conferta* after recognition of agar oligosaccharides was clearly located at the plasma membrane and it was sensitive to DPI at sub-micromolar concentrations, suggesting that an NADPH oxidase is involved in the production of ROS in this species. This idea is further supported by the sensitivity of the oxidative burst response to quinacrine and chlorpromazine, which are known to inhibit flavoenzymes such as NADPH oxidase (Auh and Murphy 1995, Bellavite et al. 1983).

Apoplastic peroxidases are excluded as enzymatic source of ROS because the H$_2$O$_2$ release in *G. conferta* after elicitation with agar oligosaccharide was clearly not located in the cell wall and not inhibited by SHAM, KCN, and NaN$_3$. The two later compounds even increased the accumulation of H$_2$O$_2$ in the culture medium, probably due to their inhibitory effect on apoplastic haloperoxidases, which are of major importance for H$_2$O$_2$ scavenging in *Gracilaria* (Pedersen et al. 1996, Weinberger et al. 1999).

Agar oligosaccharides are oxidized by *Gracilaria chilensis*. In *G. chilensis*, repeated supply of agar oligosaccharides immediately resulted in production of H$_2$O$_2$. A refractory state was not observed in this species, suggesting that agar oligosaccharide played a role as a substrate for the production of H$_2$O$_2$ rather than a signal. This idea is further supported by our finding that the site for oxygen activation was the apoplas and that proteins capable of transferring electrons from agar oligosaccharide upon the phenazine methosulfate–MTT system are present in *G. chilensis*. The H$_2$O$_2$ release response was relatively insensitive to pharmacological inhibitors. Chlorpromazine and quinacrine fully inhibited it, which points to a flavoenzyme-catalyzed reaction. A full inhibition was also observed after application of relatively low doses of SHAM, which is known to interfere with the mode of action of various metalloenzymes (O’Brien et al. 2000). KCN and NaN$_3$, two well-known inhibitors of heme and copper enzymes, also inhibited the response.

An enzymatic oxidation of agar oligosaccharide occurred, because aldehydes accumulated in the medium of *G. chilensis* when it was incubated in the presence of agar oligosaccharide. This accumulation was not correlated with an enrichment of carbohydrates and thus was not due to an excretion of aldehydes but to an oxidation of the agar oligosaccharide. Moreover, crude protein extracts of *G. chilensis* also generated aldehyde from agar oligosaccharide. Their activity was correlated with the intensity of agar oligosaccharide-dependent staining of native PAGE gels, indicating that both effects were due to the same enzyme.

Approximately 0.85 mol aldehyde was generated per mol of agar oligosaccharide during 7 h of incubation of *G. chilensis*, suggesting that every substrate molecule may only be oxidized in one specific position. This position is probably not the anomeric C-atom. An oxidation at the anomeric C-atom would result in immediate internal esterification and formation of

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**Fig. 9.** Quantification of agar oligosaccharide oxidase activity potential in crude protein extracts from two different strains of *Gracilaria chilensis*. (A) During 2 h of incubation with agar oligosaccharide substrate, two extracts from strains CUA (●) and CR14 (■) generated at approximately constant rates 10.8 ± 0.5 and 1.4 ± 0.4 μM aldehyde per h and mg protein, respectively, before the activity leveled off. No such production was observed when the same extracts were tested without substrate (* *) or when the substrate was tested without protein extract (*). (B) A linear correlation (Spearman r$^2$ = 0.853, significant at P < 0.0102) between agar oligosaccharide oxidation activity potential in crude protein extracts during 2 h and the intensity of agar oligosaccharide oxidase staining on native PAGE gels.

H$_2$O$_2$ release was possible only 6 h after the first challenge of *G. conferta* with agar oligosaccharide, indicating a new availability of unphosphorylated protein kinase substrate.

All the inhibitors of plasma membrane calcium channels that were tested (e.g. bepridil, verapamil) inhibited the release of H$_2$O$_2$ at relatively low concentrations, and an increased release was observed when the calcium ionophore A23187 was applied. An intracellular increase in the concentration of calcium ions is thus necessary in *G. conferta* to allow for oxygen activation after recognition of agar oligosaccharides, as in spermatophytes (Doke and Miura 1995, Navazio et al. 2002) and in brown seaweeds (Küpper et al. 2001) after chemosensory perception. Ophiobolin A also partially reduced the release of H$_2$O$_2$, indicating that the cytosolic release of calcium after elicitation is amplified by the calmodulin system (Hidaka and Ishikawa 1992). The inhibitor of potassium channels, 4-aminopyridine, prevented the response of *G. conferta* to agar oligosaccharides, suggesting that potassium channels also play an essential role in the process.

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All the inhibitors of plasma membrane calcium channels that were tested (e.g. bepridil, verapamil) inhibited the release of H$_2$O$_2$ at relatively low concentrations, and an increased release was observed when the calcium ionophore A23187 was applied. An intracellular increase in the concentration of calcium ions is thus necessary in *G. conferta* to allow for oxygen activation after recognition of agar oligosaccharides, as in spermatophytes (Doke and Miura 1995, Navazio et al. 2002) and in brown seaweeds (Küpper et al. 2001) after chemosensory perception. Ophiobolin A also partially reduced the release of H$_2$O$_2$, indicating that the cytosolic release of calcium after elicitation is amplified by the calmodulin system (Hidaka and Ishikawa 1992). The inhibitor of potassium channels, 4-aminopyridine, prevented the response of *G. conferta* to agar oligosaccharides, suggesting that potassium channels also play an essential role in the process.

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enzyme present in osaccharides (Avigad et al. 1962). However, the tant D-galactose at the nonreducing end than to mono-
higher affinity to oligo- and polysaccharides that con-
D-galactose and 1,5-anhydrogalactitol, but it has a
1.1.3.9). This copper enzyme acts on the C6 atom of
this enzyme are reminiscent of galactose oxidase (EC
oxidized agar oligosaccharides. The characteristics of
oligosaccharides larger than neoagarobiose (Table 2)
G. chilensis
is extremely variable, which is not only in-
indicated by the results obtained through PAGE but also
by electron microscopic observations. Distinct bands of
cerium peroxide appear in the cell wall, which presum-
represent “waves” of the enzyme that were excreted into the apoplast at different occasions. Inter-
expression of agar oligosaccharide oxidase proved to be suitable
agarase can generate these saccharides. There is no
indication for a presence of agarase in Gracilaria or other agarophytes, which leaves cell wall macerating
microorganisms as the only source. It is therefore rea-
sonable to suspect a role of agar oligosaccharide oxida-
defense of G. chilensis against agar hydrolysis.
The expression of agar oligosaccharide oxidase in G. chilensis is extremely variable, which is not only indi-
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