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FINAL REPORT

DPE 5542 -G-SS- 8042, Biotechnological evaluation of phycocolloid quality for marine algal improvement, 9/88 - 8/91

Trainees

At Berkeley

9/88-6/89 Hilconida Calumpong, while UCB Ph D. student and SUML, Assistant Director

10/89-10/90 Fraulein Cabanag, Instructor, Silliman University, Chemistry Dept.

10/90-3/91 Mirasol Magbanua, Instructor, Silliman University, Biology Dept.

4/91-8/91 Erwinia Solis-Duran, Lecturer, Silliman University, Biology Dept. & Researcher, SUML

At SUML

Fraulein Cabanag, 11/90 to present
Mirasol Magbanua, 4/91 to present
Paulina Suarez, 1/91 to present (Instructor, Chemistry Dept.)
Erwinia Solis Duran, 9/91 to present

Collaborative Visits

Hilconida Calumpong to Berkeley, November 1989 (1 month), June 1990 (1 month), June 1991 (1 week).

Valerie Vreeland to SUML and Cebu Seaweed Industries, April 1990 (2 weeks) MCPI, shemberg & FMC visited, both offices and processing factory tours, 3 seminars given and seaweed and carrageenan samples collected, accompanied by Jessica Onate, SUML researcher.

Valerie Vreeland to SUML, 3 Cebu industries and 3 farms (2 weeks, 4 seminars given), January 1991. Accompanied by Fraulein Cabanag to Cebu industries, FMC intertidal farm at Tindog Beach, Northern Cebu, and Danajon Reef farms of Shemberg and MCPI. Live plants collected and returned for culture to SUML, and carrageenan samples of characterized gel proper-ties collected.

Erick Ask, FMC Mandaue City, Cebu to SUML for seaweed culture consultation, February 1991.

Valerie Vreeland 2 weeks in January 1992. Cebu City RP-US Second International Phycological Symposium (1 paper presented) and 1 week workshop given to ten Philippine scientists at the RP-US Phycological Workshop at the Silliman University Marine Laboratory.

Electrophoresis Laboratory Established at SUML

All equipment and supplies needed to set up a functional electrophoresis laboratory were purchased, transferred to SUML and successfully used for carrageenan analysis. Equipment included a Corning still, polyacrylamide electrophoresis apparatus, power source, rotating shaker, heating block, magnetic stirrer, microcentrifuge, low-speed centrifuge, light box, polaroid camera and apparatus for gel photography. Due to an unusually hard water source, a nanopure water purification system is being purchased and added downstream from the still.

In addition to elctrophoresis, tissue printing and bacterial carrageenase technology and materials have been successfully transferred to SUML.

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Publications

Vreeland, V., Magbanua, M., Cabanag, F., Duran, E., and Calumpong, H. C. 1992. Immunolocalization of carrageenan components in seaweeds. In: Tissue Printing, Tools for the study of anatomy, histochemistry and gene expression. eds. R. Pont-Lezica, P. Reid, E. del Campillo & R. Taylor. In Press.

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Vreeland, V. 1991. Alginate and carrageenan subunit analysis with specific markers and electrophoresis. Bamfield Contemporary Macroalga products and Processes Workshop Proceedings, Vancouver. In Press. (April 20-21, invited presentation).

Abstracts

Vreeland, V., and W. M. Laetsch, 1991. Phycocolloid subunit analysis. Abstracts of the Annual American Chemistry Society Meeting, Atlanta. (Invited symposium talk, Biopolymers Symposium).

Gretz, M. R., Wu, Y., Vreeland, V., and Scott, J. 1990. Iota-carrageenan biogenesis in the red alga Agardhiella subulata is golgi mediated. J. Phycol. 26 (Suppl.) 14.

Vreeland, V., Zablackis, E., Gretz, M., Stanley, N., Stancioff, D., Kirkpatrick, F. H. and Laetsch, W. M. 1989. Electrophoretic analysis of carrageenan substructure and interactions. International Seaweed Symposium, Vancouver.

Vreeland, V., Zablackis, E., and Laetsch, W. M. 1989. Monoclonal antibodies identify intracellular synthesis of carrageenan subunits. International Seaweed Symposium, Vancouver.

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Vreeland, V., M. Magbanua, E. Duran, F. Cabanag and H. Calumpong. 1992. Tissue prints in rapid screening of carrageenan composition. Abstracts of the Second RP-US Phycological Symposium, Cebu City, the Philippines, January 6-10, 1992.

Vreeland, V., E. Zablackis, H. Calumpong, F. Cabanag, E. Duran and M. Magbanua. 1992. Electrophoretic analysis of carrageenan substructure. Abstracts of the Second PR-US Phycological Symposium, Cebu City, the Philippines, January 6-10, 1992.

Calumpong, H., V. Vreeland, E. Duran, M. Magbanua, F. Cabanag, and P. Saurez. 1992. Molecular analysis of phycocolloid quality from Philippine strains of Eucheuma and Kappaphycus. Proc. International Seaaweed Symposium 14.

TERMINAL REPORT: CARRAGEENAN PROJECT - PHILIPPINE COMPONENT

SUBMITTED BY : DR. HILCONIDA P. CALUMPONG
PHILIPPINE COLLABORATOR
SILLIMAN UNIVERSITY MARINE LABORATORY
DUMAGUETE CITY 6200 PHILIPPINES

TRAINING

The following worked at the University of California with Dr. Valerie Vreeland on various aspects of method development in carrageenan research:

Sept 1988-May 1989: Hilconida P. Calumpong, botanist: electrophoresis, tissue culture, titration studies

Oct 1989-Oct 1990: Fraulein Cabanag, chemist: electrophoresis; isolation of standards

Oct 1990-Mar 1991: Mirasol Magbanua, microbiologist: carrageenase enzymes; electrophoresis

Apr 1991-Aug 1991: Erwinia Duran, marine biologist: tissue printing and immunolabelling; electrophoresis

All of the above-mentioned personnel returned to the Silliman University Marine Laboratory to work on Philippine samples of carrageenan-producing algae from farms and industries. Paulina Suarez, another chemist from Silliman University trained on electrophoresis at the SU Marine Lab. Results of such work were presented during the 2nd RP-USA Phycology Symposium/Workshop held at Cebu City and Dumaguete City on Jan 6-18, 1992.

EQUIPMENT ACQUISITION AND LABORATORY SET-UP

Electrophoresis equipment and chemicals, including a water distiller and deionizer were purchased from the United States and sent to SUML. An electrophoresis laboratory was set-up. SUML provided laboratory space with air-con unit, refrigerator, shelves and lighting fixtures. Other equipment essential to the project, (e.g., mettler balance, microscopes) were provided by SUML.

Final Report - Fraulein Cabanag

October 4, 1990

1. Carbohydrate Transfer to Nylon Trans(+) Membrane

A. Capillary Transfer

Electrophorese carbohydrate samples in 33% polyacrylamide gel to separate the different fragments. Place the electrophoresed gel on 3 sheets of 3 mm paper prewetted with buffer and surround with parafilm to ensure that the transfer buffer goes though the gel and not around it. Carefully place a piece of Nylon trans(+)membrane on top of the gel making sure that the surface is completely covered. Roll a clean pipette over the membrane to remove air bubbles. Place 10 sheets of dry 3 mm paper, roll a pipette over the sheets, cover with a glass plate and a 1 kg weight. Allow the transfer to proceed for 2 hours.

B. Vacuum Transfer

Assemble the vacuum transfer apparatus ensuring that the rubber mat is cut such that the gel overlaps the window by about 5mm. Place an appropriate membrane size under the rubber mat so that it is exactly positioned within the window of the mat. Transfer the gel accurately on the membrane and avoid trapping air bubbles between the gel and membrane. Turn on the house vacuum pump at full position to immobilize the gel and carefully place about 5-10 ml of buffer on top of the gel. Make sure that the top of the gel is always wet with buffer. Allow the transfer to proceed for 2 hours.

C. Graphite Plate Electrotransfer

Wet graphite plates with dd water and wipe off excess before wetting with the transfer buffer. On the anode graphite, place the following in the proper order: 10 sheets of 3 mm paper prewetted with transfer buffer, one sheet of nylon trans(+) membrane, the gel, 3 sheets of 3 mm sheets prewetted with buffer, and then the cathode graphite. Make sure that no air bubbles are trapped between the gel and the membrane. Allow the transfer to proceed for two hours at 0.8 mA per cm^2 gel.

2. Staining of Transfer Membranes

Incubate the membrane in 1% Tween 20 solution for one minute and immediately stain with 0.1% Fluka Alcian Blue for 5 minutes. Wash several times with water until the background becomes light. This method of staining is possible for at least 4 sulfate groups on the carbohydrate (DP 4 for kappa and perhaps DP 2 for iota) as evidenced by neocarrahexaosetetrasulfate staining and not neocarrabiose-4-sulfate, neocarratetraose-4-sulfate and neocarratetraosedisulfate. The sensitivity of staining is about 50 ng.

3. Fractionation of Kappa and Iota Carrageenan Using QAE Sephadex at 70 and 90°C with 2.25 and 3.5 M Imidazole (pH=7.0)

Weigh 1 mg each of SK and SI, dissolve in 500 ul of water and add to 450 ul of pre-soaked QAE Sephadex beads and let stand overnight. Wash the incubated beads with water several times to

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remove unabsorbed carbohydrate. To the washed beads add 1.0 ml of 2.25M imidazole and heat at 70°C in a multi-blok heater overnight. Withdraw the supernatant, wash the beads three times with 1.0 ml hot water (70°C), combine washings with the supernatant, and add 3 volumes of $CaCl_2$-EtOH. To the washed beads, add 1.0 ml of 3.5M imidazole and heat at 90°C overnight. Separate the supernatant and rinse the beads three times with 90°C water and combine washings with the supernatant. Treat the supernatant in the same manner as above. Note: Kappa fragments are completely eluted from the beads at about 2.25M imidazole and iota at 3.5 M based on the liquid chromatographic separation of kappa and iota fragments using imidazole as the eluant.

4. HCl Hydrolysis and Fractionation of Long Kappa/Iota Fragments Using 2.5 M KCl and EtOH

Weigh 0.500 gms of Long Kappa/Iota, dissolve in water to a total volume of 75 ml, heat the solution at 80°C with stirring until all particles are dissolved. Adjust the pH to 2 using 0.5 M HCl and heat at 80°C in a water bath for 30 minutes , cool and neutralize with 0.5 M KOH. Adjust the volume to 100 ml, add 450 ml of 2.5 M KCl and let flocculate for 30 minutes and centrifuge. The precipitate contains the longest fragments. To the supernatant add 100% EtOH to make a total concentration of 30% and let flocculate for another 30 minutes. The precipitate contains mostly long fragments and intermediate ones. To the supernatant add 100% EtOH to make a 40% alcohol solution . Let flocculate for 30 minutes. The precipitate contains more of the intermediate fragments and a little of the shortest fragments. To the supernatant add EtOH to make the concentration of alcohol 50% and let flocculate overnight at room temperature to prevent KCl crystallization. Centrifuge at 25° . The precipitate contains the shortest fragments. The shortest fragments are treated with 0.5M HCl in ice bath to prevent degradation and exchange K^+ ions bound to the carrageenan with H^+ ions from the acid for one hour. Hydrogen ions have greater affinity than potassium ions thus solid ion exchange takes place.

5. Ion Fractionation of Kappa/Iota Fragments Using 2.5M KCl, $Mg(NO_3)_2$, $CaCl_2$ and NaCl

Five hundred microliters of 10 mg/ml short kappa/iota is added to 13x100 test tubes each containing 4.5 ml of KCl, $Mg(NO_3)_2$, $CaCl_2$ and NaCl. Let stand for 30 minutes and centrifuge. Add 100% EtOH to make 30% solution and let flocculate. Separate the precipitate and add 100% alcohol to the supernantant to make 40% and flocculate. Separate the precipitate and add again alcohol to make a total concemtration of 50% alcohol and flocculate. Note: Results showed that for KCl fractionation, both kappa and iota short fragments are precipitated at 50% EtOH. Only the longest fragments of iota are precipitated with NaCl only or NaCl-EtOH while shorter kappa fragments are precipitated better at lower concentration of alcohol (about 30%). Long and short kappa fragments are precipitated with only Mg and Mg-EtOH. For iota, only the longest fragments are precipitated with any concentration of alcohol and a trace of the shorter fragments with only Mg. Mostly long fragments of iota are precipitated with Ca

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EtOH and long fragments and a trace of shorter ones for Ca only. Most of the long and short fragments of kappa are precipitated with only Ca and as the concentration of EtOH is increased, the longer gragments disappear and only shorter ones are precipitated.

6. Neocarrahexaosetetrasulfate as marker for Carrageenans

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Final Report - Mirasol Magbanua, March 1991

Recovery of carrageenases from cultures of <u>Pseudomonas carrageenovora</u> (K-ase) and Bacteria 1 (I-ase):

1. Media preparation:
   weigh 37.4 grams of Bacto marine Broth 2216 (from Difco laboratories), and suspend in 1,000 ml of distilled water. Heat to boiling for 1 to 2 minutes until dissolved.
   a. Pour about 30 ml of medium into a separate flask., then divide the remaining medium (about 970ml) into 2 portions. Label 1 as K-ase and the other as I-ase.

   b. To the K-ase flask, add .25% kappa carrageenan; to the I-ase flask, add .25% iota carrageenan.

   c. To the 30 ml of medium, take 10 ml and add 1% agarose, The remaining 20 ml is divided into equally into 2 small flask.

   d. Cover all flask with cotton plugs and autoclave at 15 pounds of pressure, 115 degrees centigrade, for 15 minutes.

   e. After autoclaving, immediately pour medium with agarose into [illegible] sterile plates. Label 1 plate <u>P.carrageenovora</u> and the other plate Bacteria 1.

2. Batch culture preparation:

   a. Using the Marine agar plates prepared (1d), and frozen bacterial cultures, inoculate each plate with the respective bacteria using the streak plate method and incubate for 24 hours.
   b. Pick out an isolated colony from the 24 hour plate culture and transfer to the flask with 15 ml of Bacto marine broth without carrageenan (1c). Be sure to label flask with the correct bacterial name. Incubate 24 hours at 23 degrees centigrade.
   c. Transfer the 24 hour culture of <u>P.carrageenovora</u> into the flask labelled K-ase with kappa carrageenan, and the culture of Bacteria 1 into the flask labelled I-ase contanining iota carrageenan.Incubate 24 hours.

   note; all incubations are at 23 degrees centigrade with continous shaking.

   d. Take 1ml from the 24 hour culture with carrageenan and freeze in cryotubes at -80 degrees centigrade.
   e. Enzymes are harvested from the remainder of the cultures with carrageenan.

3. Harvesting of enzymes:

   a. Harvest all at 4 degrees centigrade.
   b. Spin bacterial cells out at 10,000 rpm for 40 minutes.
   d. To the supernatant, add ammonium sulfate up to 70% saturation to precipitate the enzymes. Allow to shake in the cold room overnight.
   e. Centrifuge at 15,000 rpm for 40 minutes. Collect the precipitate.
   f. Dialyze with buffer (25 mM sodium phosphate ph 6.5 for k-ase, and 25 mM sodium phosphate in 2 m NaCl for I-ase, since I-ase is relatively unstable and needs to be stabilized with NaCl.

CARRAGEENASE GEL:

Gel preparation:
Melt 1% agarose in 50 mM Tris ph 7.5 and imM EDTA. Add .1% carrageenan. Heat to boiling to dissolve the agarose. Pour into the mold at approximately 55 degrees centigrade.

ENZYME ACTIVITY TEST:

1. Prepare the gel mold. Clean glass plates & spacer. The gel bond should adhere closely to the plate (avoid bubbles).
2. Pour the gel and allow to solidify for about 30 minutes.
3. Remove the glass plates, gel should be adherent to gelbond.
4. A 1 microliter sample is loaded onto the gel surface,
5. Incubate gel for 1hour .
6. Wash gel for 30 minute with 200 mM NaCl and 10 mM EDTA.
7. Stain with 1% Fluka Alcian blue for 20 minutes(gel should be covered when in alcian blue)
8. Wash the gel with water and blot dry with filter paper and paper towels.

PROTEASE GEL:

Gel preparation:
Melt 1% agarose in 50 mM Tris ph 7.5 and 1 mM EDTA. Add .1% Casein (Casein is dissolved in KOH to ph 11 - 12, then adjusted with 6M HCL to ph 8-9, final ph is 8.5)

PROTEASE ACTIVITY TEST:

Procedure is the same as the enzyme activity test, except for the staining procedure.

1. Prepare gel mold. Clean glass plates and spacer. The gel bond should adhere closely to the plate (avoid bubbles).
2. Pour the gel and allow to solidify for about 30 minutes.
3. Remove the glass plates, gel should be adherent to gelbond.
4. A 1 microliter sample is loaded on to the gel surface and incubated for 1hour.
5. Gel is washed with 1% acetic acid for 30 minutes.
6. Stain gel with Coomassie brilliant blue stain (1mg/ml, dissolved in Meoh, HoAC and H20, 5:1:4). for about 5 minutes.
7. Destain with MeOH,HoAC, and H20, 5:1:4.
8. Wash with water and then blot dry with filter paper and paper towels.

note; incubation for both carrageenase and protease gels were done at 35 to 40 degrees centigrade.

ALKALI MODIFICATION OF EUCHEUMA sp.

1. Sort and clean algae (separate haploid and diploid plants where possible).
2. Chop samples into small pieces then wash with 95% ethyl alcohol until clear (about 30 minutes).
3. Wash with acetone once, then dry a few minutes.
4. Add 500mM KCl and 50 mM CaCl2 (enough to cover samples) and sodium borohydride (NaBH4, about 1 mg/ml). Mix and allow to stand overnight.
5. The next day, add more NaBH4 and 3M KOH (approximately equal volume), then heat at 80 degrees centigrade for 7 to 8 hours.
6. Wash out alkali with water briefly.

EXTRACTION OF CARRAGEENAN FROM ALKALI MODIFIED EUCHEUMA SP.

(Based on the Handbook of Phycollogical methods,by Johan A. Hellebust and J.S.Craigie, modified by Vreeland)

1. Chop samples as fine as possible, wash with acetone and allow to settle. Remove supernatant, then repeat the process.
2. Add 0.5 M NaHCo3, stir at 90 degrees centigrade for 2 hours to extract carrageenan.
3. Precipitate carrageenan by slow addition with stirring of Hyamine 1% in alcohol and water. (2% aqueous Cetylmethylammonium bromide - Cetavlon).
4. Wash precipitate with distilled water twice.
5. Wash repeatedly on the centrifuge (up to 7 times) with near saturated solution of sodium acetate in 95 % ethanol (200 mM sodium acetate in 95% ethanol/in water, 4:1) Stir to break up clumps.
6. At 3 final washings with warm 95% ethanol are required to remove the sodium acetate.
7. Wash with acetone and dry overnight.

AMMEDIOL _ TRICINE _ POLYACRYLAMIDE GEL ELECTROPHORESIS

CLUB SANDWICH GEL:
Weigh 11.5 grams of acrylamide and 0.25 grams of piperazine diacrylamide (PDA), dissolved in 25 ml of 200mM ammediol-HCL buffer (ph 8.79) and dilute to 50ml with water (this is equal to 23% acrylamide and 0.5% PDA). Filter on a vacuum flask and degas for 15 minutes. Add 115 ul of 10% ammonium persulfate (APS) and 60 ul of TEMED, swirl 6-8 times. Withdraw the solution using a syringe and pour the liquid as quickly as possible without forming bubbles until the solution is level with the top of the glass plates.

TOP BUFFER:
Weigh 6.272 grams of tricine and 3.679 grams of ammediol and dissolve in water and dilute to 350 ml (equivalent to 100mM ammediol and 100mM tricine).

BOTTOM BUFFER:
Weigh 4.208 grams of ammediol and dissolve in about 100ml of water. Adjust the ph to 8.79 with 6M HCL and dilute to 200 ml (equivalent to 200mM ammediol-HCL). 25 ml of this buffer is used to prepare the gel. The remaining buffer is diluted to 350 to make 100mM buffer.

SAMPLE PREPARATION:
A stock solution (10mg/ml is prepared by weighing 10 mgs of carrageenan and dissolved in 1 ml of water. 2 microliters of the stock solution is diluted to 20 microliter with 0.25 M sucrose solution with sodium azide, to make a 1 mg/ml solution. Solutions are stored in the freezer.

STAINING OF THE GEL:
Gel is stained with 0.1% alcian blue (weigh 0.2grams of alcian blue and dissolve in 200 ml of water, this solution is good for 1 gel). Immerse the gel in alcian blue solution and cover the basin with aluminum foil to protect from light. Mix on the rotating shaker for 30 minutes. Wash the gel several times with water and occasionally rub surface of gel with wet kimwipe to remove precipitate until the background is clear.

OXIDATION:
Prepare a stock solution of the oxidizing agent by weighing 4.79 grams pf potassium dichromate,dissolve in about 100ml of water. Add 1 ml of concentrated nitric acid (specific gravity, 1.42) and dilute to 500ml. Store in the refrigerator. 20 ml of the solution is diluted to 200ml, enough to oxidize 1 gel. The gel is incubated for 5 minutes in the solution, then washed several times with water for 30 minutes until gel is completely clear.

SILVER STAINING OF GEL:
A stock solution is prepared by weighing 10.2 grams of silver nitrate, dissolve in 100ml of water and diluted to 500ml. This is stored in the refrigerator. 20 ml of the solution is diluted to 200 ml for staining 1 gel. The gel is incubated for 20 minutes in this solution. After incubation, the gel is immersed briefly in water without agitation. Prolonged washing will remove the silver reagent and result to weak staining.

STAIN DEVELOPMENT:
Prepare the developer stock solution by weighing 27.7 grams of sodium carbonate decahydrate, dissolve in about 100ml of water, add o.5 ml of 37% formaldehyde solution or paraformaldehyde and dilute to 1 liter. Put the gel under a white background, then add about 50 ml of the developer. Shake during development and replace with fresh developer when brown precipitate appears. When the background starts to darken, stop development by immersing the gel in5% acetic acid for 5 minutes. Wash gel with water several times and store in ziploc bags.

ANTIBODY LABELLING OF CARRAGEENAN TISSUE BLOTS AND DOT BLOTS.

SOLUTIONS:

1. Ovalbumin (1%) is dissolved with stirring in 50mM Tris, ph 7.5 with 0.12% sodium azide. The solution remains cloudy. Centrifuge to clarify at 12,000+G. Aliquot and freeze.

2. Second antibody: Alkaline phosphatase conjugate of anti-mouse kappa light chains (Southern Biotechnology, purchased through Fisher). Dilute 1:1000 in hybridoma medium containing 20% serum.

3. Enzyme substrate: Add 0.75ml BCIP stock 4mg/ml (60 mg BCIP + 10ml MEOH + 5 ml acetone store at -20 degrees centigrade) and 5 mg of NBT(nitro blue tetrazolium,stored dry in the refrigerator) to 50 ml of Tris 0.2M + 4mM MgCl2, ph 9.5 (Tris stock: 24.2g Tris + 810mg MgCl2.H2O dilute to 1 liter with H2O).

ANTIBODY LABELLING PROTOCOL:

1. Incubate blots in 1% centrifuged ovalbumin for 15 minutes.
2. Wash with salt solutions (200mM NaCl + 10mM CaCl2) for about 1 minute.
3. Incubate 100 ul per cm2 of antibody solution to the membrane for 15 minutes.

   10A5 (5/3/90 #12) precursor
   3G1 (3/10/88 #8) Lambda
   4D12 (7/28/87) Iota
   6A11 (9/14/88) kappa
   7C2 (9/22/88 #13) gelling
   7E4 (9/14/88 #24) gelling
   7H6 (5/3/90 #30) gelling

4. Wash each one separately with the salt solution twice (about 2 minutes each, use the shaker to remove all traces of unbound antibody).
5. Incubate in second antibody for 15 minutes.
6. Wash with salt solution twice 1-5 minutes.
7. Incubate in enzyme substrate for 5 to 15 minutes until background starts to increase Stop substrate development by washing in a large volume of water.
8. Allow enough time to dry. (Result is better when membrane has dried).

FINAL REPORT
of Erwinia Solis-Duran

SUMMARY OF METHODOLOGIES DEVELOPED FOR CARRAGEENAN ANALYSIS

A. Enzyme Hydrolysis of Carrageenan:

Take 20 ul of enzyme (k-ase and i-ase) and put in 2-ml tube. Spin for 5 minutes then decant liquid. Add 1 mg/ml of carrageenan, shake to mix then incubate at 37 C overnight.

Take 30 ul of sample from the stock and return for another overnight incubation. Add sucrose to sample and run an electrophoresis.

B. Acid Hydrolysis of Carrageenan

Weigh sample and dissolve in water to make 0.67%. Heat to complete dissolution. Cool then adjust pH to 2 with 0.1M HCl. Heat for 3 minutes at 80 C and immediately cool in ice bath. xxNeutralize with 0.1M NaOH. Add 3 volumes of isopropanol, shake to mix, let stand overnight. Centrifuge for 5 min. then decant isopropanol. Heat for 15 min. to dry precipitate completely. Sample may be stored in dry form in the freezer or with 0.25 M sucrose equivalent to 1 mg/ml and this would be ready for electrophoresis.

C. Tissue Blot and Immunolabelling of Diploid *Chondrus*

a. Tissue blot
1. Soak plants in 200 mM NaCl for 30 min.
2. Roll plant crosswise then make thin sections using a clean, sharp blade.
3. Blot thin sections on a nylon membrane for 30 sec. to 1 min. on the slide warmer heated to 70 C.
4. Remove the sections from the membrane then allow to dry.

b. Immunolabelling with 3G1 (a lambda antibody)
1. Incubate membrane in 1% ovalbumin for 15 min.
2. Wash in 200mM NaCl + 10 mM CaCl salt solution
3. Incubate in 3G1 antibody for 15 min.
4. Wash in salt solution
5. Incubate in second antibody diluted in hybridoma medium (1: 1,000) for 15 min.
6. Wash in salt solution
7. Incubate in enzyme substrate until background starts to increase.
8. Wash in water for 2 min.

D. Elution of Kappa Bands from a Preparative Gel:

1. Prepare .1% TBO and 250mM Tetramethylammonium (TMA) chloride
2. Run a preparative gel (23% ammediol-tricine acrylamide) with acid hydrolyzed pure kappa as sample

3. Incubate electrophoresed gel in .1% TBO for 5 min. on shaker. Wash in water until bands are seen.
4. Lay the electrophoresed gel on a glass or clear plastic plate placed on top of a light box. Note the arrangement of bands in proximity to the color markers.
5. Number each visible band with the bottom most band as 1. Usually there are 11 bands that are visible and can be separated from other bands.
6. Cut each band with a scalpel and remove unwanted gel or space in between bands. Macerate gel with band or cut into small pieces to reduce surface area.
7. Put the macerated band in a small glass container with cover containing 4 mls of TMA chloride.
8. Add blue rayon and another 4 mls to each container then leave overnight on the shaker. Blue rayon absorbs the TBO.
9. Once the liquid and gel have become clear, remove the liquid with a pipet using the blue rayon as filter.
10. Put the liquid in a 50ml tube and add 2 volume of isopropanol and NaCl to a final concentration of 100mM. Let stand in cold room for 1 hr.
11. Transfer into 2ml tubes then spin to precipitate the carbohydrate. Decant until precipitate is completely dry.
12. Add little amount of distilled water (0.5ml) and blue cotton then leave overnight on the shaker.
13. Take out the blue cotton and add 3 volume of isopropanol.
14. Spin to precipitate carbohydrate. Decant alcohol then add a tiny amount of water to the precipitate and sucrose. This will be ready for electrophoresis.

E. Reducing end assay for enzyme hydrolyzed carrageenan samples

1. Preparation of working reagents, stock solutions A & B
   Stock solution A: 97.1 mg of disodium 2,2'-bicinchoninate (Sigma Chemical) dissolve in 45 ml of water containing 3.2 g of sodium carbonate monohydrate and 1.2 g sodium bicarbonate. Adjust volume to 50ml.
   Stock solution B: 62 mg of copper sulfate pentahydrate and 63 mg of L-serine dissollved in 45 ml of water. Adjust final volume to 50 ml.
2. Mix equal volumes of solutions A and B.
3. Add 100 ul of enzyme-hydrolyzed (i.e. 24-hr incubated, 36-hr, etc.) samples without sucrose to 100ul of working reagent in the wells of a 96-sample microtiter plate. Cover plate with Saran wrap and incubate in a water bath at 80 C for 35 min. Cool for 15 min then measure the absorbance at 560 nm. Do triplicate analyses for each sample.

G. Fast and sensitive silver staining of polyacrylamide gels:

1. Fix electrophorese gel in 0.1% Fluka Alcian Blue for 30 min., on a shaker, covered with aluminum wrap.
3. Wash gel with water while shaking. Wipe gel with kimwipe from time to time until gel becomes clear.
4. Imregnate gel with AgNO3 (1g/li), 1.5 ml 37%

HCOH/liter for 30 min.afterwhich rinse in water without shaking for not more than 1 min.
5. Develop geel in Na_2CO_3 (30g/li), 1.5 ml of 37% HCOH/li, $Na_2S_2O_3 \cdot 5H_2O$ (2mg/li) for 2-5 min.
6. Stop gel development in 10 % acetic acid for 5 min.

H. Alkali modification of live *Gymnogongrus furcellatus*, a kappa and iota producer

For cottonii (kappa):

1. Wash samples briefly in 95% ethyl alcohol to wash off epiphytes, about 2 dips.
2. Add 0.5N KOH + 1.5M KCl
3. Heat for 3 hours at 85 C.
4. Soak in 2 M NaCl to wash off alkali for 4 times at 15-30 min. each.
5. Do tissue blots then antibody labelling.

For spinosum (iota):

1. Wash samples briefly in 95% ethyl alcohol to wash off epiphytes, about 2 dips.
2. Add 250 mM $CaCl_2$ + 500 mM KOH + 1.5 M KCl
3. Heat for 15 min at 85 C.
4. Soak in 2M NaCl to wash off alkali 4 times at 15-30 min each wash.
5. Do tissue blots then antibody labelling.

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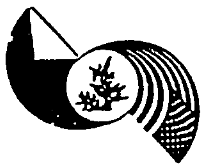

2nd RP-USA Phycology Symposium/Workshop

Symposium: Cebu Plaza, Cebu Ctiy, 6-10 January 1992
Workshop: Silliman University, Dumaguete City, 11-18 January 1992

ABSTRACTS

Theme: *Contemporary concepts and method in systematics, biology and ecology of marine algae, including seagrasses in the Philippines.*

Sponsored by: The US National Science Foundation

•SUPPORTING AGENCIES: Department of Foreign Affairs-Office of International Cooperation for Science and Technology • Department of Agriculture-Bureau of Fisheries and Aquatic Resources • Department of Environment and Natural Resources-Environmental Bureau• Department of Science and Technology-Philippine Council for Aquatic and Marine Research and Development• Office of the President-Technology and Livelihood Center • Seaweed Information

TISSUE PRINTS IN RAPID SCREENING OF CARRAGEENAN COMPOSITION.

Valerie Vreeland[1], Mirasol Magbanua[2], Erwinia Duran[2], Fraulein Cabanag[3] and Hilconida Calumpong[2].

* Presented paper at Cebu Symposium

[1]Department of Plant Biology, University of California, Berkeley, CA 94720, USA; Departments of [2]Biology and [3]Chemistry, Silliman University, Dumaguete, the Philippines.

A rapid and sensitive assay was developed for determining the carrageenan composition of individual plants. A thin section of a branch from each plant was cut and briefly pressed onto a membrane. After the alga section was discarded, membrane-bound carrageenan in tissue impressions was labeled by panel of eight monoclonal antibodies. Multiple samples were labeled in a in one-hour assay. The assay is modified for Extracted carrageenan samples were labeled in dot blots. Dot blots can be quantitated by scanning densitometry. Each antibody is specific for a different epitope or short sequence of sugar units found in carrageenans. One epitope is closely related to kappa carrageenan, another to lambda carrageenan, another to iota carrageenan, and another to precursor of kappa or iota. The remaining four epitopes were present in kappa and iota carrageenans to varying degrees.

Each antibody labeled a series of carrageenophytes from Danajon Reef in a different pattern. The kappa-related antibody labeled all *Kappaphycus alvarezii* - *Eucheuma cottonii*, which are harvested for kappa carrageenan, but not *Eucheuma denticulatum* - *E. spinosum*, which are harvested for iota carrageenan. The lambda-related antibody labeled only iota-producers. The precursor antibody labeled the iota-producing plants strongly and some kappa-producers more weakly. Tissue prints of *Chondrus crispus* sporophytes were labeled primarily by the lambda-related antibody, as expected.

Tissue printing with the anti-carrageenan monolconal antibodies easily differentiated kappa- and iota-producing plants. The results show mixed carrageenan composition in some plants. Tissue printing can be used to select individual plants with relatively pure kappa or iota content for seedling material or for certain applications. Tissue printing was also used to detect carrageenan presence and composition in food products.

ELECTROPHORETIC ANALYSIS OF CARRAGEENAN SUBSTRUCTURE.

Valerie Vreeland[1], Earl Zablackis[2], Hilconida Calumpong[3], Fraulein Cabanag[4], Erwinia Duran[3] and Mirasol Magbanua[3].

[1]Department of Plant Biology, University of California, Berkeley, CA 94720, USA; [2]Complex Carbohydrate Research Center, University of Georgia, 220 Riverbend Road, Athens, GA 30602 USA; Departments of [3]Biology and [4]Chemistry, Silliman University, Dumaguete, the Philippines.

Carrageenan structure is complex and related to its useful properties. Rapid comparison of carrageenan substructure in multiple samples was accomplished using polyacrylamide electrophoresis. Minimum carrageenan quantity needed for electrophoresis is 2-5 micrograms, and the minumum size of fragments fixed and detected have 3-4 sulfate groups. Kappa, iota and lambda carrageenan samples were fragmented by acid hydrolysis and resolved by electrophoresis. Ladders consisting of discrete bands were produced by kappa and iota fragments. Each band corresponds to fragments of a unique size, differing in length by one disaccharide repeat. Iota produces both a wider band spacing and faster migration compared to kappa at the same chain length, due to the higher sulfate content of iota. Kappa and iota preparations each have one major band and one or more minor bands in the repeat pattern. Lambda carrageenan from nonreproductive diploid *Chondrus crispus* sporophytes did not produce a band pattern. Instead, a smear was stained in the short-fragment region of the gels. This provides evidence for a lack of regular repeating structure in lambda carrageenan.

Kappa and iota carrageenan samples were fragmented by specific enzymatic hydrolysis for analysis of composition and native subunit size. Kappa and iota samples were hydrolyzed with both kappa carrageenase (*Pseudomonas carrageenovora*) and iota carrageenase (Bacterium 1). Kappa segments were typicaly in a relatively short size range in kappa carrageenan, but iota segments were considerably longer in iota carrageenan. This difference is expected to be related to gelation mechanism and gel properties. A small amount of short iota fragments was detected in kappa carrageenan. This finding supports the occurrence of hybrid kappa-iota molecules. Electrophoretic analysis can be used to compare small samples from many individual plants.

Report in a Philippine newspaper in January 1992 during the Second Republic of the Philippines-United States Phycology Workshop

Thursday, January 9, 1992 THE FREEMAN 19

PHYCOLOGY SYMPOSIUM AT CEBU PLAZA: The 2nd RP-USA Phycology Symposium is currently going on until Jan. 10 at Cebu Plaza Hotel. After the symposium, the participants will move on to Silliman University in Dumaguete City for a week-long workshop. Shown in photo are (from left): Dr. Hilconida Calumpong, director, Silliman University Marine Laboratory; Janet Estacion and Erwinia Duran, also from SU Marine Lab; Dr. David Ballantine, resource person; Roy de Leon; Dr. Valerie Vreeland and Dr. Lynda Goff, resource person; CPH PR Assistant Rachel Zabala; and Dr. Ernani Meñez, director, Smithsonian Institution Oceanographic Sorting Center in the U.S. (CPH PR)

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6. Benjamin T. Que
Mindanao Lumad and Muslim Development Center
Xavier Science Foundation, Inc.

Sun Star Daily / Tuesday

Sun·Star Business
Editor: FRED C. ESPINOZA

RP, U.S. scholars establish national seaweed program

Group to introduce new knowledge

By FRED C. ESPINOZA of Sun Star Daily

THE first USA-Philippines Phycology Workshop in 1984 has blazed the trail for the establishment of a full-fledged national seaweed program for the country ...

SCHOLARS

Nat'l seaweed program set up by scholars

From Page B-1

Dr. Hilconida Calumpong, director of the Silliman University marine laboratory, ... project of Dr. Ronald Phillips (formerly with Seattle Pacific University) ...

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Second International RP-US Phycology Symposium in Ceby City, 1/92

Benson Dakay, President of Shemberg Marketing (the largest Philippine Seaweed processing and farming company); Dr. Hilconida Calumpong, Director of the Silliman University Marine Laboratory (and co-investigator of the AID project); Dr. Ernani Menez, Smithosinan Institution, Washington, D.C. (organizer of the symposium and workshop, as well as mentor to the AID project).

Dr. Valerie Vreeland, Berkeley co-investigator; Vicente Alvarez, FMC Marine Colloids, founder of the Philippine seaweed farming industry; Erwinia Duran, Silliman Marine Laboratory instructor and lecturer, Philippine trainee for AID.

Carrageenan Biotechnology Workshop at Dumaguete

Valerie Vreeland. University of California. Berkeley. and Hilconida Calumpong. Fraulein Cabanag. Mirasol Magbanua. Erwinia Duran and Paulina Saurez. Silliman University. Dumaguete.

This workshop provided a demonstration of carrageenan analytical methods recently developed during the A.I.D. project. It consisted of five full days in the Silliman Univerisity Marine Laboratory. The number of participants was ten, coming from the Philippine seaweed industry, goverment agencies and college and university teachers. Tissue printing. carrageenan electrophoresis and carrageenase bacterial culture and activity detection were demonstrated by Fraulein Cabanag and Dr. Vreeland., Dr. Vreeland lectured for 4-5 hours on these subjects. For three days. the participants carried out tissue printing and electrophoresis, working in two groups of five each.

Tissue printing for rapid evaluation of carrageenan quality in farmed plants with monoclonal antibodies. This includes selection of plant material, tissue printing, antibody incubations. image development. and interpretation of results. In addition. dot blots for qualitative analysis of dissolved carrageenans were demonstrated.

The participants were each given aliquots of all eight anti-carrageenan monoclonal antibodies on ice for use in their own laboratories.

Carrageenase fragmentation by enzymatic and acidic hydrolysis was discussed. including microscale carrageenan extraction and recovery. as well as acidic and enzymatic hydrolysis of carrageenan.

Kappa- and iota-carrageenase preparation. Inoculation of medium from frozen bacteria, culture of carrageenase-producing bacteria. removal of bacterial cells was demonstrated. Carrageenase activity on substrate gels was demonstrated, and all participants were given the two bacterial strains which produce kappa and iota carrageenases.

Polyacrylamide electrophoresis **of carrageenan fragment types. Sample preparation, polyacrylamide gel preparation, sample loading, electrophoresis, gel staining and interpretation of results.**

CARRAGEENAN TISSUE PRINT AND ELECTROPHORESIS WORKSHOP
Silliman University Marine Laboratory
January 13-17, 1992

Republic of the Philippines - US
Second Phycology Symposium/Workshop

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Five day workshop on Carrageenan analysis at the Silliman University Marine Laboratory during the Second RP-US Phycological Workshop

The ten Philippine participants with Dr. Vreeland (blue skirt) and Fraulein Cabanag (purple shirt), the Aid trainee who carried out the laboratory demonstrations.

Fraulein Cabanag, AID trainee and lecturer in the Chemistry Department at Silliman University, conducting an electrophoresis demonstration for a group of participants.

Workshop participants Purita Balean (college instructor), Annabelle Briones (government scientist) and Dr. Marcos Montano (university professor) doing tissue printing of carrageenan-producing plants.

Workshop participants Editha Gamboa (government scientist), Farley Baricuatro (industrial scientist) and Grace Prado (university instructor) doing electrophoresis of carrageenan fragments.

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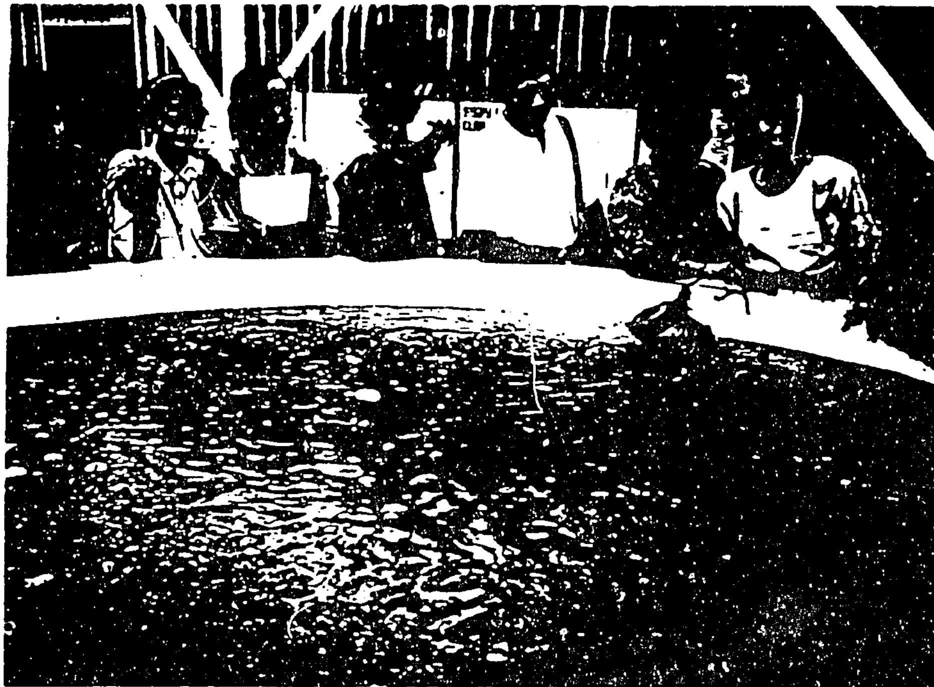
Workshop participants Margie Legaste, (industry), Grace Prado (academic), Elnor Roa (academic), Farley Baricuatro (industry), Reyh Paspie (industry), Purita Balean (academic) and Annabelle Briones (government) examining seaweeds in a culture tank at Silliman University Marine Laboratory, Dumaguete City.



Carrageenan-producing plants used for tissue printing during the workshop. The large-diameter plants are from a government-sponsored educational test farm at San Jose, a few miles north of Dumaguete, and the smaller-diameter plants were collected by Dr. Vreeland next to the Marine Colloids Philippines International platform on Danajon Reef during a field trip earlier in the symposium.

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SUML

CARRAGEENAN TISSUE PRINTS

SOLUTIONS

1. Ovalbumin (1%) is dissolved with stirring in 50 mM Tris, pH 7.5 with 0.12% sodium azide. The solution remains cloudy and some is not dissolved. Centrifuge to clarify at 12,000+G. Aliquot and freeze.

2. Second antibody: alkaline phosphatase conjugate of anti-mouse 'kappa light chains (Southern Biotechnology, purchased through Fisher). Dilute 1:100 in hybridoma medium containing 20% serum.

3. Enzyme substrate. Add 0.75 ml BCIP stock 4 mg/ml (60 mg BCIP + 10 ml MEOH + 5 ml acetone, store at -20°C) and 5 mg of NBT (nitro blue tetrazolium, stored dry in the refrigerator) to 50 ml of Tris 0.2M + 4Mm $MgCl_2$, ph 9.5. (Tris stock: 24.2g Tris + 810 mg $MgCl_2.H20$ dilute to 1 liter with dH_2O.

IN SITU ALKALI MODIFICATION

For cottonii (kappa):
1. Wash samples briefly in 95% ethyl alcohol to wash off epiphytes, about 2 dips.
2. Add 0.5N KOH + 1.5M KCl
3. Heat for 3 hours at 85 °C.
4. Soak in 2 M NaCl to wash off alkali for 4 times at 15-30 min. each.
5. Do tissue blots then antibody labelling.

For spinosum (iota):
1. Wash samples briefly in 95% ethyl alcohol to wash off epiphytes, about 2 dips.
2. Add 250 mM CaCl2 + 500 mM KOH + 1.5 M KCl
3. Heat for 15 min at 85 °C.
4. Soak in 2M NaCl to wash off alkali 4 times at 15-30 min each wash.
5. Do tissue blots then antibody labelling.

ANTIBODY LABELING

A. Incubate in 1% centrifuged ovalbumin for 15 min.

B. Wash with 200 mM NaCl + 10 mM $CaCl_2$ for 1 min. Remove excess liquid.

C. Incubate membrane in 50 ul per cm^2 of one anti-carrageenan antibody in hybridoma medium solution for 15 min.

D. Wash each membrane separately with 200mM NaCl + 10 mM $CaCl_2$

for 2 minutes twice.

E. Incubate second antibody for 15 minutes.

F. Wash with salt solution twice 1-5 min.

G. Incubate in substrate for 5-15 minutes until background starts to increase. Stop substrate development by washing in a large volume of water. Addition of 50% ethanol to the wash removes substrate that can increase background during storage.

H. View print with a dissecting microscope or a magnifying lens. If print is not completely dry, artifactual dark lines will be seen in depressions caused by cell walls.

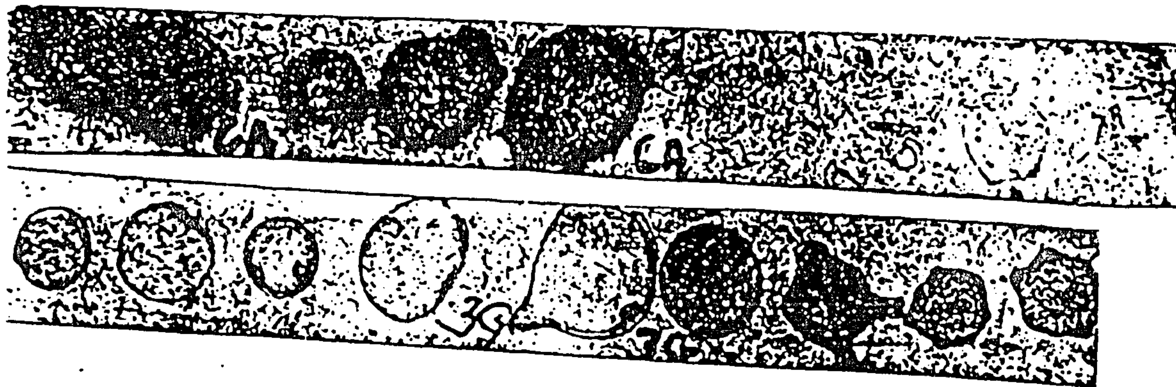
PHOTOGRAPHY OF STAINED PRINTS

Photograph prints enlarged by a dissecting microscope or use a camera with close-up attachments. Different effects are achieved with ring-lighting above and with low-angle side-lighting.

Technical Pan Estar-based film (Kodak) provides high contrast images. Bracket ASA 100-200 (ASA 125-160 is optimal).

Develop in HC-110 developer dilution B (473 ml concentrate diluted to 1.9 L for the stock solution, then dilute 1:7 stock:water). Alternatively, use 9.3 ml concentrate per 300 ml to develop one roll.
Development time: 8.5 min at 22-23°C room temperature for high contrast.
Stop bath: 30 sec. in 1% glacial acetic acid, with agitation.
Fixer: 5 min in Kodak ektafix, diluted 75 ml/ 300 ml.
Wash: 1 min in running water.
Fixer removal: 30 sec in Hustler hypo clearing agent with agitation, 9 ml/300 ml.
Wash: 5 min in running water.
Prevent water spots on negatives: 1-2 ml/300 ml photoflow in tank water for 2-3 min.
Air-dry negatives.

As an alternative to photography, the prints can be enlarged while copying for a permanent record.



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Recovery of Carrageenases from Cultures of *Pseudomonas carrageenovora* (K-ase) and Bacteria 1 (I-ase)

1. MEDIA PREPARATION

Weigh 37.4 grams of Bacto marine Broth 2216 (from Difco laboratories), and suspend in 1,000 ml of distilled water. Heat to boiling for 1 to 2 minutes until dissolved.

a) Pour about 30 ml of medium into a separate flask, then divide the remaining medium (about 970 ml) into 2 portions. Label 1 as K-ase and the other as I-ase.

b) To the K-ase flask, add 0.25% kappa carrageenan; to the I-ase flask, add 0.25% iota carrageenan.

c) To the 30 ml of medium, take 10 ml and add 1% agarose. The remaining 20 ml is divided equally into 2 small flasks.

d) Cover all flasks with cotton plugs and autoclave at 15 pounds of pressure, 115 degrees centigrade, for 15 minutes.

e) After autoclaving, immediately pour medium with agarose into sterile plates. Label 1 plate *P. carrageenovora* and the other plate Bacteria 1.

2. BATCH CULTURE PREPARATION

a) Using the Marine agar plates prepared (1d), and frozen bacterial cultures, KBInoculate each plate with the appropriate frozen culture by streaking the plate directly from the surface of the frozen culture. Incubate for 24 hours.

b) Pick out an isolated colony from the 24 hour plate culture and transfer to the flask with 15 ml of Bacto marine broth without carrageenan (1c). Be sure to label flask with the correct bacterial name. Incubate 24 hours at 23 degrees centigrade.

c) Transfer the 24 hour culture of *P. carrageenovora* into the flask labelled K-ase with kappa carrageenan, and the culture of Bacteria 1 into the flask labelled I-ase containing iota carrageenan. Incubate 24 hours.

Note: All incubations are at 23 degrees centigrade with continuous shaking.

d) Take 1 ml from the 24 hour culture with carrageenan and freeze in cryotubes at -80 degrees centigrade.

e) Enzymes are harvested from the remainder of the cultures with carrageenan.

3. Harvesting of Enzymes:

a) Maintain temperature at 4 $^{\circ}$ C with an ice bath.

b) Spin bacterial cells out of 10,000 rpm for 40 minutes.

c) To the supernatant, add ammonium sulfate up to 70% saturation to precipitate the enzymes. Allow to shake in the cold room overnight.

d) Centrifuge at 15,000 rpm for 40 minutes. Collect the precipitate.

e) Dialyze with buffer (25 mM sodium phosphate ph 6.5 for K-ase, and 25 mM sodium phosphate in 2 m NaCl for I-ase, since I-ase is relatively unstable and needs to be stabilized with NaCl.

CARRAGEENASE ACTIVITY GEL

Gel Preparation: Melt 1% agarose in 50 mM Tris ph 7.5 and 1 mM EDTA. Add 0.1% carrageenan. Heat to boiling to dissolve the agarose. Pour into the mold at approximately 55 degrees centigrade.

1. Prepare the gel mold. Clean glass plates and spacer. The gel bond should adhere closely to the plate (avoid bubbles)

2. Pour the gel and allow to solidify for about 30 minutes.

3. Remove the glass plates, gel should be adherent to gelbond.

4. A micrliter sample is loaded onto the gel surface.

5. Incubate gel for 1 hour.

6. Wash gel for 30 minutes with 200 mM NaCl and 10 mM EDTA

7. Stain with 1% fluka Alcian Blue for 20 minutes (gel should be covered when in alcian blue)

8. Wash the gel with water and blot dry with filter paper and paper towels.

Note: incubation for carrageenase gels were done at 35 to 40 degrees centigrade.

CARRAGEENAN HYDROLYSIS FOR ELECTROPHORESIS

A. ENZYMATIC HYDROLYSIS

1. Put 20 ul of ammonium sulfate-precipitated enzyme (k-ase or i-ase) in a 2-ml tube. Centrifuge enzyme suspension for 5 minutes at 13,000 g. Carefully remove liquid with a pipettor tip and discard liquid.
2. Add 1 ml of 1 mg/ml of carrageenan in enzyme buffer (25 mM Na_2PO_4, pH 6.5 for k-ase and the same buffer with 100 mM NaCl for i-ase. Shake to mix. Incubate at 37 ^{0}C overnight.
3. Repeat the overnight incubation with 20 ul of fresh enzyme precipitate.
4. Add 3 volumes of isopropanol, shake to mix, let stand overnight. Centrifuge at 13,000 g for 5 min. then remove isopropanol with a pipette tip. Heat at 50-70 ^{o}C for 15 min. to dry precipitate completely.
5. For electrophoresis of enzymatically hydrolyzed samples, add 0.25 vol of 1 M sucrose and use without removal of enzyme.

B. ACIDIC HYDROLYSIS OF CARRAGEENAN

1. Make a 5 mg/mL solution of carrageenan in water. Heat to dissolve completely, and then cool.
2. Adjust pH to 2 with 0.1M HCl. Heat at 80 ^{0}C for 30 minutes and immediately cool in an ice bath. Neutralize to pH 7 with 0.1M NaOH.
3. Sample may be stored in dry form at room temperature, or in the freezer when dissolved.

August 10, 1990

AMMEDIOL-TRICINE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Club Sandwich Gel
Weigh 11.5 grams of acrylamide and 0.25 grams of piperazine diacrylamide, dissolve in 25 ml of 200mM ammediol-HCl buffer(pH=8.79 and dilute to 50 ml with water (equivalent to 23% acrylamide and 0.5% PDA). Filter on a vacuum flask and degas for 15 minutes. Add 115 ul of 10% ammonium persulfate and 60 ul of TEMED, swirl 6-8 times. Withdraw the solution using a syringe and pour the liquid as quickly as possible without forming bubbles until the solution is level with the top of the glass plates.

Top Buffer
Weigh 6.272 grams of tricine and 3.679 grams of ammediol and dissolve in water and dilute to 350 ml (equivalent to 100mM ammediol and 100mM tricine).

Bottom Buffer
Weigh 4.208 grams of ammediol and dissolve in about 100 ml of water. Adjust the pH to 8.79 with 6M HCl and dilute to 200 ml (equivalent to 200mM ammediol-HCl). Twenty five milliliters of this buffer is used to prepare the gel. The remaining buffer is diluted to 350 ml to make 100 mM buffer.

Sample Preparation
A stock solution (10 mg/ml) is prepared by weighing 10 mgs of dried pectate and dissolve in 1 ml of water and kept in the freezer. Two microliters of the stock solution is diluted to 20 ul with 0.25 M sucrose solution (with sodium azide) to make 1mg/ml. This solution could be used 6 times since the usual amount of sample used for pectates is only 3 ug.

Staining
Weigh 0.2 grams of Alcian Blue and dissolve in water and dilute to 200 ml to make 0.1% solution. This solution is good for one gel only. Immerse the gel in the solution and cover the basin with aluminum foil to protect the stain from light and mix slowly on the rotating shaker for 30 minutes. Wash the gel several times with water and occasionally rubbing the surface of the gel with a wet kimwipe to remove the precipitate until the background is clear.

Oxidation
A stock solution of the oxidizing agent is prepared by weighing 4.79 grams of potassium dichromate, dissolve in about 100 ml of water, add 1 ml of conc. nitric acid (specific gravity of 1.42) and dilute to 500 ml. This solution is stored in the fridge. Twenty milliliters of this solution is diluted to 200 ml for oxidizing one gel. The gel is incubated for 5 minutes in this solution and washed several times with water for about 30 minutes until the gel is completely clear.

ELUTION OF BANDS FROM PREPARATIVE GELS

1. Prepare 0.1% TBO and 250mM Tetramethylammonium (TMA) chloride.
2. Run a preparative gel (23% ammediol-tricine acrylamide) with acid hydrolyzed pure kappa as sample
3. Incubate electrophoresed gel in .1% TBO for 5 min. on shaker. Wash in water until bands are seen.
4. Lay the electrophoresed gel on a glass or clear plastic plate placed on top of a light box. Note the arrangement of bands in proximity to the color markers.
5. Number each visible band with the bottom most band as 1. Usually there are 11 bands that are visible and can be separated from other bands.
6. Cut each band with a scalpel and remove unwanted gel or space in between bands. Macerate gel with band or cut into small pieces to reduce surface area.
7. Put the macerated band in a small glass container with cover containing 4 mls of TMA chloride.
8. Add blue rayon and another 4 mls to each container then leave overnight on the shaker. Blue rayon absorbs the TBO.
9. Once the liquid and gel have become clear, remove the liquid with a pipet using the blue rayon as filter.
10. Put the liquid in a 50ml tube and add 2 volume of isopropanol and NaCl to a final concentration of 100mM. Let stand in cold room for 1 hr.
11. Transfer into 2ml tubes then spin to precipitate the carbohydrate. Decant until precipitate is completely dry.
12. Add little amount of distilled water (0.5ml) and blue cotton then leave overnight on the shaker.
13. Take out the blue cotton and add 3 volume of isopropanol.
14. Spin to precipitate carbohydrate. Decant alcohol then add a tiny amount of water to the precipitate and sucrose. This will be ready for electrophoresis.

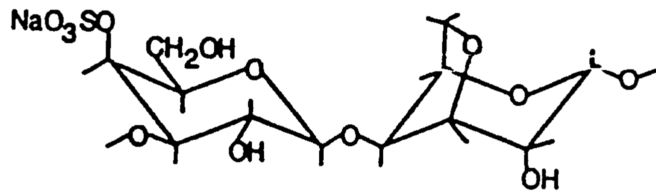
Silver Staining

A stock solution is prepared by weighing 10.2 grams of silver nitrate, dissolving in about 100 ml of water and diluted to 500 ml and kept in the fridge. Twenty milliliters of this solution is diluted to 200 ml for staining one gel. The gel is incubated for 20 minutes in this solution. After incubation the gel is immersed in water for one minute to remove excess reagent without agitation. Prolonged washing will remove silver reagent from the gel and staining will be weak or lost.

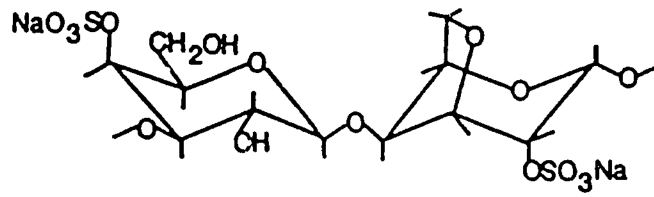
Stain Development

A developer solution is prepared by weighing 27.7 grams of sodium carbonate decahydrate, dissolve in about 100 ml of water, add 0.5 ml of 37% formaldehyde solution or paraformaldehyde and dilute to 1 liter. Put the gel under a white background before adding about 50 ml of the developer. Shake during development and replace developer with a fresh one when a brown precipitate appears. The stained bands would come slowly and development should be stopped when the background starts to darken by immediately removing the developer and immersing gel in 5% acetic acid for 5 minutes. Wash the gel with water several times before storing in ziploc bags.

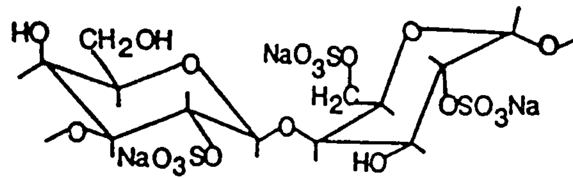
CARRAGEENAN



Kappa



Iota



Lambda

α(1→3)–D galactose β(1→4)–D–galactose

Table 2. Antibody specificities by enzyme immunoassay inhibition. Relative amounts of kappa, iota and lambda carrageenans needed for 50% reduction of antibody binding to kappa carrageenan, compared to a value of "1" for the best inhibitor.

Antibody	µg/well[1]	long kappa	long iota	Lambda	short kappa	short iota
6A11-2A4	1.1(κ)	1	50	60	>9	>9
5H12	0.03(κ)	1	3.6	600	1.6	8.8
7E4-3E9	0.6(κ)	1	2.3	>33	3.7	>33
10A5	1.1(κ)	1	1.6	69	1.8	8.6
7C2	0.03(κ)	1	1.7	233	38	1,296
7H6-3D12	0.3(κ)	1	1.1	16	2	16.1
4D12-1A12	0.4(ι)	2.9	1	1.9	>345	9
3G1	0.4(λ)	11.9	2.3	1	153	4.9
3G1 + DL[2]		109	30			

[1] microrgrams of antigen needed per well of a microtiter plate for 50% inhibition by the best inhibitor, either long kappa (κ), long iota (ι) or long lambda (λ).

[2] diploid Chondrus lambda was used both to coat the plate (instead of long kappa) and as inhibitor.

XIVth INTERNATIONAL SEAWEED SYMPOSIUM

ABSTRACT FORM

Brittany France 16 - 21 August 1992
Université de Bretagne Occidentale
Symposium Secretariat
Institut d'Etudes Marines
29287 BREST Cédex
FRANCE

Sample Abstract

Shivji, M.S.* & R.A. Cattolico. University of Washington, Seattle, W.A. USA. ORGANIZATION OF CHLOROPLAST GENOMES IN RED SEAWEEDS.
Seaweed chloroplast genomes remain relatively unstudied, partly due to difficulties in purification of undegraded organellar DNA from these polysaccharide rich algae. Information on algal chloroplast genome structure and gene content is required to elucidate...

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TYPE ABSTRACT IN SPACE BELOW
Instructions on back of this page and spacing of sample abstract above

Calumpong, H.,*[1], V. Vreeland, [2] E. Duran, [1] M. Magbanua, [1] F. Cabanag[1] & P. Saurez[1]. [1]Silliman University, Marine Laboratory, Dumaguete, The Philippines, & [2]University of California, Berkeley, CA, USA.

MOLECULAR ANALYSIS OF PHYCOCOLLOID QUALITY FROM PHILIPPINE STRAINS OF *EUCHEUMA* AND *KAPPAPHYCUS*.

Tissue printing and electrophoretic analysis were used for rapid comparison of small samples from many plants. Carrageenan epitopes recognized by eight monoclonal antibodies were detected by tissue printing of seven strains of *Eucheuma* and *Kappaphycus*. Kappa- and iota-producing plants were readily differentiated within two hours. Antibody 6A11 to a kappa-related epitope labeled only *Kappaphycus* and antibody 3G1 to a lambda-related epitope labeled only *Eucheuma*. Mixed carrageenan composition was found in some plants. Tissue prints can aid in seedling selection and dot blots can detect carrageenan presence and composition in products. Extracted carrageenan was fragmented by enzymatic hydrolysis and the size distributions of kappa and iota sequences were compared by oligosaccharide electrophoresis. Fragment size distributions, which varied among samples, are expected to be correlated with gel properties. Supported by AID Grant DPE-5542-G-SS-8042.

Projection Requirements (others than 35mm slide projector or overhead projector).

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Submission Category
- Invited paper ____
- Contributed paper X
- Poster ____

If we are unable to schedule your talk, would you be willing to give a poster?
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I would like to compete for Best Student awards (see page 8)
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____

Signature of Supervisor / Head of Department. A letter of verification should be also enclosed. (see page 8)

P05A Identification of genomic clones encoding a wound-induced cell wall protein (p33) in carrot.

B. Ebener, J. Shaver, M.L. Tierney. Agronomy Department and Biotechnology Center, Ohio State University, Columbus, Ohio 43210- 1002.

p33 is a proline-rich cell wall protein which is induced by wounding in carrot storage roots. This protein is a member of a family of structural cell wall proteins which are synthesized in response to developmental and wound-induced signals. p33 mRNA and protein have been shown to accumulate rapidly in carrot roots after wounding, indicating that the synthesis of this cell wall protein is one of the early cellular responses to physical damage. In order to identify regulatory factors responsible for the synthesis of p33 in wounded tissue, it is necessary to characterize the promoter sequences of genomic clones encoding this protein. Toward this end, we have constructed a carrot genomic library in lambda GEM 11 (Promega) and have identified 9 genomic clones which hybridize to pDC16, a wound-induced p33 cDNA clone. To further characterize these clones we have synthesized specific oligonucleotides based on the p33 cDNA sequence. When these oligonucleotides are used in polymerase chain reactions (PCR) using a p33 cDNA as the template, a 500 base pair DNA fragment is generated which contains an asymetric Sca1 site. PCR analysis of the p33-related genomic clones, using these primers, indicated that several clones also gave rise to a 500 base pair fragment containing a Sca1 site. DNA sequence analysis of the PCR products will be used to confirm that these genomic clones are identical in sequence to the wound-induced p33 cDNA. Once genomic clones encoding the wound-induced p33 protein have been identified they will be used to characterize the cis-acting DNA sequences involved in regulating p33 expression in wounded tissue.

P06B Cell Wall Biogenesis in the Red Alga Agardhiella: A Simple Model System for Studies of Extracellular Matrix Assembly.

Michael R. Gretz and Yalin Wu, Dept. Biology, George Mason University, Fairfax, VA 22030. Valerie Vreeland, Dept. Plant Biology, University of California Berkeley, CA 94720 and Joe Scott, Dept. Biology, College of William & Mary, Williamsburg, VA 23185.

The extracellular matrix of many marine red algae is composed of two major components, cellulose and a sulfated galactan. We have demonstrated the presence of iota-carrageenan (3-linked-ß-D-galactose-4-sulfate alternating with 4-linked-3,6-anhydro-α-L-galactose-2-sulfate residues) and cellulose in *Agardhiella subulata* by ^{13}C-NMR, IR, methylation analysis and micro-chemical techniques. Ultrastructural immunogold localization with an anti-carrageenan monoclonal antibody demonstrated carrageenan distribution throughout the cell wall of cortical areas in apical regions. Intracellular deposits of anti-carrageenan/gold were restricted to trans Golgi cisternae and associated vesicles. Cellobiohydrolase/colloidal gold labelled wall areas and gave no indication of intracellular ß-4-glycan. Preliminary evidence suggests carrageenan deposition precedes cellulose biogenesis in *A. subulata* apical cells and that biosynthesis of these two polymers is separated spatially as well as temporally. The two-polysaccharide cell wall of *Agardhiella* should serve as a simple model system for the study of biosynthesis and assembly of the plant extracellular matrix.

Biopolymers Symposium
ACS meeting, Atlanta, April 1991

The Controlled Release of a Nerve Growth Factor Protein from a Chitosan Matrix

G.G. Allan and J.G. Winterowd, Department of Forest Resources, 304 Bloedel, University of Washington, Seattle, WA 98195.

Channels (6 mm in length) composed of a blend of 37% N-acetylated chitosan (1 mg) and nerve growth factor (NGF) protein (0.3 μg) were suitable as a guidance and NGF sustained delivery system for the regeneration of severed mouse sciatic nerves. The NGF protein had no statistically significant influence on the regenerated nerves. The channels seemed to elicit an inflammatory response in the mice and were substantially eroded after six weeks. When films made of chitosan and radioactively labeled NGF protein were immersed in aqueous solutions of lysozyme (pH value = 7.4, T = 37°C) it was found that 15-70% of the protein in the film was eluted immediately and the remainder was eluted in a manner which could be explained by a simple diffusion model ($D = 1.2 \times 10^{-10}$ cm^2/s).

Chitosan-Copper Complexes

B.L. Averbach[1], M.J. Condon[1], and M. Nakashima[2], [1]Department of Material Science, Massachusetts Institute of Technology, Cambridge, MA 02139 and [2]Soldier Science Directorate, U.S. Army Natick RD&E Center, Natick, MA 01760-5020.

Copper chitosan (copper-[N-acetyle-D-glucosamine]) complexes were prepared in two ways. In one, the copper ion was added as copper acetate to a chitosan-acetic acid solution from which membranes were cast. In the second, chitosan membranes were cast from chitosan-acetic acid solutions and then soaked in dilute solutions of copper sulfate. X-ray diffraction data indicated that the copper acetate membranes were amorphous. The copper sulfate membranes were crystalline, with indications that crystallites of copper sulfate and metallic copper were present. XPS spectra showed that the copper was bonded as a metallic complex in the copper-acetate membranes. The bonding in the copper sulfate membranes was also that of a metallic complex, but copper peaks corresponding to those in copper-sulfate were also observed.

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Phycocolloid Subunit Analysis

V. Vreeland and W.M. Laetsch, Department of Plant Biology, University of California, Berkeley, CA 94720.

The subunits of gelling carbohydrates from marine plants (carrageenan, agar and alginate) and other sources (pectate, xanthan) were investigated with rapid, sensitive, microscale methods. Subunit size distribution was analyzed by electrophoresis into discrete bands after specific enzymatic cleavage. Subunit types were identified and localized in tissue sections and on membrane blots by molecular markers. Molecular markers prepared included monoclonal antibodies with specificities for gelling, nongelling and precursor subunits, as well as direct gelling probes. Intracellular synthesis and differential subunit distribution in cell walls was found for the phycocolloids. A type of nongelling organization of alginate gelling subunits was found, and evidence supporting a nongelling role for alginate in adhesion of brown algal embryos to the substrate was produced. Differences in pectate distribution, esterification and molecular size were found during fruit ripening. This approach is being used for phycocolloid detection and quality assessment. This research was supported by NSF, California Sea Grant College Program, Kraft, Marine Colloids and the Agency for International Development.

Synthetic Antifreeze Peptides

T. Caceci, M.B.W. Szumanski, T.E. Toth, and J.A. Cobb, Virginia/Maryland Regional College of Veterinary Medicine, VPI&SU, Blacksburg, Virginia 24061.

For many years it has been known that certain species of cold water fishes elaborate polypeptide and glycoprotein "antifreezes" as a protection against intraorganismal ice formation. These materials are capable of suppressing the growth of ice crystals to temperatures as low as -2° Celsius. The peptide antifreezes have been well studied. Native antifreeze peptides (AFPs) are rigid rod-shaped molecules composed of a limited variety of amino acids, principally alanine and threonine in the case of class I (alanine-rich) forms. The combination of polar and nonpolar amino acids, the internal and external dipole moments, and the orientation of the active (hydrophilic) groups on one side of the rod permit the molecules to orient correctly and hydrogen-bond to the forming ice crystal face. Active groups are spaced to facilitate this bonding. We have designed and produced synthetic AFPs twice the size of natural ones, and are testing them for activity below -2°C.

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SEASONAL VARIATION OF PHOTOSYNTHESIS, GROWTH AND BIOMASS OF GIGARTINA PECTINATA (RHODOPHYTA) IN THE GULF OF CALIFORNIA. Alejandro Cabello, Isai Pacheco, Jose A. Zertuche and Boudevijn H. Brinkhuis. State Univ. of New York at Stony Brook and Instituto de Investigaciones Oceanologicas, Ensenada, Mexico.

Photosynthesis, respiration, growth and percent cover of the commercially valuable seaweed, Gigartina pectinata, was examined for approximately two years. This species in endemic to the Gulf of California and it was found growing in the high sublittoral zone to a maximum depth of 6 m. In the study zone it appears during winter and disappears in mid-summer. A maximum daily in situ growth of 10.6% was recorded at the begining of spring and a maximum biomass of 398.5 ± 119.0 g dry weight m^{-2} was found at the end of spring. Growth in the field is negatively affected by high temperature during the summer months. Laboratory growth was strongly reduced at irradiances higher than 113 $\mu E\ m^{-2}\ s^{-1}$ and temperatures above 26°C. This species seems to grow optimally at irradiances between 50 and 100 $\mu E\ m^{-2}\ s^{-1}$ and temperatures between 18 and 22°C.

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IOTA-CARRAGEENAN BIOGENESIS IN THE RED ALGA *AGARDHIELLA SUBULATA* IS GOLGI MEDIATED. Michael R. Gretz and Yalin Wu, Dept. Biology, George Mason University, Fairfax, VA 22030. Valerie Vreeland, Dept. Botany, University of California, Berkeley, CA 94720 and Joe Scott, Dept. Biology, College of William & Mary, Williamsburg, VA 23185.

The cell walls of both life cycle phases of *Agardhiella subulata* consist predominately of iota-carrageenan and cellulose as demonstrated by ^{13}C-nuclear magnetic resonance, infrared spectroscopy, methylation analysis and micro-chemical techniques. Colloidal gold/monoclonal immunocytochemical localization of iota-carrageenan demonstrated carrageenan distribution throughout the cell wall in apical regions of thalli. Intracellular deposits of anti-carrageenan/gold were restricted to trans Golgi cisterna and associated vesicles. Cellobiohydrolase/colloidal gold labelled wall areas and gave no indication of intracellular ß-4-glycan. Preliminary evidence suggests carrageenan deposition precedes cellulose biogenesis in *A. subulata* apical cells and that biosynthesis of these two polymers is separated spatially as well as temporally. The two-polysaccharide cell wall of *Agardhiella* should serve as a simple model system for the study of biosynthesis and assembly of plant cell walls.

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PORPHYRIDIUM CRUENTUM - AN ALTERNATIVE EPA PRODUCER. Zvi Cohen and Yair M. Heimer. Desert Research Institute, Ben-Gurion University, Sde-Boker ISRAEL.

The red microalga Porphyridium cruentum is a potential source for the pharmaceutically valuable fatty acids eicosapentaenoic acid (EPA) and arachidonic acid. The fatty acid composition of this alga is highly dependent on environmental conditions. A careful control of the cell concentration and growth temperature brought about increased EPA contents, both indoors and outdoors. Further improvement was achieved by strain selection. Methods developed for EPA purification resulted in an oil containing 90% EPA. Another approach involved the use of herbicides. The herbicide SAN 9785 is known to inhibit the desaturation of linoleic acid to linolenic acid. By treating P. cruentum cultures with this herbicide it was possible to obtain herbicide resistant cultures, some of which may have an increased EPA content.

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PHOTOCONTROL OF GERMINATION IN POST PRIMARY AND SECONDARY DORMANT OOSPORES OF *NITELLA FURCATA*. Roger C. Sokol. Department of Biological Sciences, Universtiy at Albany, State University of New York, Albany, N.Y. 12222.

Light was required to germinate both post primary (PPD) and post secondary dormant (PSD) oospores of *Nitella furcata* subsp. *megacarpa* (Allen emend. Wood). An action spectrum for germination of PSD oospores provides evidence for phytochrome as the photoreceptor. The PSD oospores were extremely sensitive to light and responded to photon fluences in the range of 1 x 10^{-4} to 3 x 10^{1} µmole m^{-2}. Germination was promoted by all wavelengths tested between 420 and 760 nm and was not red/far-red reversible. At the most sensitive wavelength (669 nm) a half-maximal response was obtained with a photon fluence of 0.3 µmole m^{-2}, classifying it as a very low fluence response (VLFR) of phytochrome. By contrast, PPD oospores required multiple 15 hour photocycles of red light to germinate maximally. The suggestion is that the VLFR may be a consequence of oospore banking in lake sediments. Activation of PPD oospores was up to 10 fold larger under red than under white light, which may implicate the participation of a second, blue light photoreceptor.

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THERMO- AND PHOTOPERIOD DISCRIMINATORS WITHIN A GERMINATION WINDOW OF CHARA OOSPORES: LOCATORS OF EXTERNAL BOUNDARIES. R.G. Stross, Dept. of Biol. Sciences, Univ. (SUNY) at Albany.

Germination regulators were detected in a clone of innately dormant oospores of Chara zeylanica (Klein ex. Waldenow). Grown in outdoor tanks, fully ripened oospores were transferred to various combinations of light and temperature for afterripening. Germination was measured in a standardized assay arena in response to light and temperature. A germination window opened independently of afterripening temperature. It was maximally open for less than a month, with a midpoint at 6.2 months postharvest. Within the window, germination ranged from non (0.0) in short-day to all (100%) in long-day thermocycles. The requirement for light and the controlling effect of daylength on germination was established in permissive thermocycles and in constant temperature. The controlling effect of daylength demanded the oospores be afterripened in a light/dark cycle. Long known to sense 'gaps' or "internal" boundaries of a plant canopy, propagules now give evidence of sensing "external boundaries, such as might be displayed in Nitella meadows, treelines, and the intertidal.

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The physiology of dimethylsulfoniopropionate (DMSP) production in phytoplankton.
Maureen D. Keller and Wendy K. Bellows
Both at: Bigelow Laboratory for Ocean Sciences
McKown Point, W. Boothbay Harbor, ME 04575

Dimethylsulfoniopropionate (DMSP) is the precursor of dimethyl sulfide (DMS), the primary volatile organic sulfur compound released from the world's oceans. DMS flux from the oceans is estimated currently at ~1.2 Tmol $S.y^{-1}$, or about half the amount of sulfur resulting from anthropogenic activities, and has been implicated in important global atmospheric processes. Significant production of DMSP is confined to a few classes of marine phytoplankton, primarily the Dinophyceae and Prymnesiophyceae. In these groups, DMSP can account for up to 80% of total organic sulfur. DMSP remains intracellular and fairly constant over the growth cycle until late stationary phase when extracellular levels begin to rise, suggesting leakage. We have examined the effects of a number of environmental variables on DMSP production and release in several marine phytoplankton. In particular the effects of perturbations in light, temperature and nutrient status have been determined. These results will be discussed in relation to marine sulfur chemistry, with ancillary comments on freshwater phytoplankton.

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Cloning of the Gene for VP54, the Major Capsid Protein of *Chlorella* Virus PBCV-1. Michael V Graves and Russel H. Meints, Oregon State University, Corvallis, Oregon 97331 USA.

PBCV-1 is a large polyhedral dsDNA (~330 kbp) virus which infects a unicellular, eukaryotic, exsymbiont *Chlorella*-like green algae. Structurally, the virus is 64% protein, 25% DNA, and 5-10% lipid. Large quantities of the virus can be purified from algal cultures and a plaque assay has been developed making this a novel system for studying the regulation of gene expression in a photosynthetic host-virus relationship.

The major capsid protein (MW=54 kD) of the virus, a glycoprotein which constitutes ~40% of the total virion protein, has been purified using SDS-PAGE and liquid column chromatography. This protein exists in the virus as a dimer (MW=104 kD) which is stable in the presence of SDS, urea, or a reducing agent, but can be dissociated using heat (100 °C) or phenol. A polyclonal antiserum has been raised to this protein and partial amino acid sequence data have been obtained from native protein as well as CNBr digestion fragments. Western blot analysis of proteins isolated at various time points post-infection indicates that the capsid protein is synthesized after DNA replication has begun and is therefore probably encoded by a late gene. The sequence data were used to construct degenerate oligonucleotides which were subsequently utilized via PCR of genomic DNA to amplify and clone the gene for VP54.

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ULTRASTRUCTURAL IMMUNOGOLD LOCALIZATION OF CARRAGEENAN IN THE GOLGI COMPLEX OF *AGARDHIELLA SUBULATA*. Michael R. Gretz and Yalin Wu, George Mason University, Fairfax, VA 22030; Joe Scott, College of William & Mary, Williamsburg, VA 23185; and Valerie Vreeland, University of California Berkeley, CA 94720, USA.

The extracellular matrix of many marine red algae is composed of two major components, cellulose and a sulfated galactan. We have demonstrated the presence of iota-carrageenan (3-linked-ß-D-galactose-4-sulfate alternating with 4-linked-3,6-anhydro-α-L-galactose-2-sulfate residues) and cellulose in *Agardhiella subulata* by ^{13}C-NMR, IR, methylation analysis and microchemical techniques. Ultrastructural immunogold localization with an anti-carrageenan monoclonal antibody demonstrated carrageenan distribution throughout the cell wall of cortical areas in apical regions. The only intracellular localization of anti-carrageenan/gold was over trans Golgi cisternae and associated vesicles. No indication of intracellular ß-4-glycan was shown using cellobiohydrolase/colloidal gold although wall areas were labelled. Our results suggest that carrageenan deposition precedes cellulose biogenesis in *A. subulata* apical cells and that biosynthesis of these two polymers is separated spatially as well as temporally.

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SEASONAL SUCCESSION IN A SOIL ALGAL COMMUNITY ASSOCIATED WITH A YOUNG BEECH-MAPLE FOREST IN NORTHEASTERN OHIO. Anne E. Grondin and Jeffrey R. Johansen, Department of Biology, John Carroll University, University Heights, Ohio 44118 USA.

A total of 41 algal taxa was identified from the soil of a young beech-maple forest. The taxa were distributed in the following classes as indicated: 2 Cyanophyceae, 29 Chlorophyceae, 1 Ulvaphyceae, 1 Chrysophyceae, 4 Xanthophyceae, and 5 Bacillariophyceae. Successional patterns were not very clear. Although species composition changed over time, many species were present year-round. Algal densities, as determined through dilution plate techniques, fluctuated during the year of the study, with peaks occurring in January (a brief warming period in mid-winter), May (late spring), August, and September (late summer/early fall). Algal numbers seemed most closely tied to temperature, although soil moisture was also important. The complex environmental changes that occur at this site during the year are likely responsible for the lack of clear patterns. These changes include: temperature fluctuations, unpredictable precipitation patterns, shading by tree canopy, and shading by leaf litter. This study suggests that in order to study seasonal succession in forest soils a more frequent sampling regime than once per month is needed.

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VANADATE-SENSITIVE ATPase IN THE ACIDOPHILIC RED ALGA GALDIERIA SULPHURARIA. Wolfgang Gross. Biology Dept., University of California Santa Cruz, USA. Present address: Inst. Plant Physiology. Free University Berlin, 1000 Berlin 33, Germany.

The alga Galdieria sulphuraria is a thermo- and acidophilic unicellular rhodophyte. While the pH in the growth medium can be as low as 1, the cytosolic pH remains neutral. Therefore, a proton gradient exists across the plasma membrane of about 1:10^{6}. High activity of a vanadate-sensitive ATPase was found in partially purified plasma membranes from Galdieria. The optimum of the ATPase was around pH 6.0. The activity of the ATPase at 50° C was 30 times higher than at 20° C. In contrast, various soluble enzymes showed only 5 - 6 fold increase in activity. This temperature sensitivity of the ATPase in Galdieria would be necessary to compensate for the increase in proton flux at higher growth temperatures.

Supported by the Alexander-von-Humboldt Foundation.

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GENES ENCODING LIGHT HARVESTING PROTEINS IN THE DIATOM *PHAEODACTYLUM TRICORNUTUM*. Arthur R. Grossman, Annamaria Manodori, Devaki Bhaya, Kirk Apt. Carnegie Institution of Washington, Stanford, CA, 94305, USA

The major light harvesting complex in the diatoms is a fucoxanthin, chlorophyll *a*, *c* complex. The polypeptides of this complex exhibit some immunological relatedness to the chlorophyll *a*, *b* binding (Cab) polypeptides of the major light harvesting complex in terrestrial plants. Antibodies to the fucoxanthin, chlorophyll *a*, *c* binding proteins (Fcps) were used to isolate the cognate genes. The *fcp* genes are a small multigene family on the nuclear genome of *Phaeodactylum tricornutum*. Based on gene sequences, the Fcps exhibit some homology to Cab polypeptides of higher plants, primarily in a region of the Cab polypeptides thought to be important for chlorophyll binding. Furthermore, Fcps are synthesized as precursor polypeptides in the cytoplasm of the cell. In terrestrial plants the presequence, or transit peptide, is essential for the post-translational targeting of the protein to the chloroplast. In the diatoms the presequence is more similar both in sequence and biochemical characteristics to a signal sequence, which is involved in the cotranslational transport of proteins into the endoplasmic reticulum. These results are discussed with reference to the evolution of plastids in the chromophytic algae.

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PRELIMINARY STUDY OF THE CHARACEAE SPECIES IN HIGH-ALTITUDE LAKES (WESTERN EUROPE, MOROCCO AND BOLIVIA). Micheline Guerlesquin, I.R.F.A.-U.C.O., B.P.808, F-49008 ANGERS Cedex 01, France

Forty-six microspecies (sensu WOOD,1965) have been gathered from a number of the lakes in eight studied altitude regions (higher than 550 m) in France (Vosges, Jura, Alps, Auvergne, Pyrenees), northern and eastern parts of Spain, Morocco and Bolivia: 26 *Chara*, 2 *Lamprothamnium*, 1 *Nitellopsis*, 14 *Nitella* and 3 *Tolypella*. The Characeae species rarely exceed 2000 m in Western Europe and Morocco. But they can be found at more than 4000 m in Bolivia.

The cosmopolitan and subcosmopolitan taxa predominate in all the studied regions (37,5 %). A large percentage of endemic species (31 %) are noticed in Bolivia in accordance with what is presently known.

At high altitude, the Characeae species are often sterile or produce frequent abnormalities in the morphology of their thalli and their gametangia. Sometimes the monoecious plants (with conjoined gametangia on the same node) seem to become subdioecious in time (exaggerated protandry, to all appearances female plants) or in space: the male and female gametangia are frequently produced at different nodes (sejoined gametangia).

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Vreeland, V.,*E. Zablackis and W.M. Laetsch. MONOCLONAL ANTIBODIES IDENTIFY INTRACELLULAR SYNTHESIS OF CARRAGEENAN SUBUNITS.

Monoclonal antibodies identified kappa, iota and lambda carrageenan structures by immunofluorescence on red algal sections. Methacrylate sections of Eucheuma alvarezii var. Tambalang, which produces kappa carrageenan, were examined. The pattern of extracellular labelling was distinctive for each antibody. The anti-kappa antibody labelled all cell walls and matrix brightly, except for the cuticle and the outermost few cell layers. The anti-iota antibody weakly labelled the matrix between walls, except near the thallus surface. The anti-lambda antibody brightly labelled only the cuticle at the thallus surface and a wall layer on a few cells in the core. All of these antibodies labelled intracellular inclusions in the 2-3 cell layers of small cells adjacent to the columnar epidermal cells, even when the walls of these cells was not labelled. Supported by California Sea Grant R/MP-36, Marine Colloids Division of FMC, Inc., and Agency for International Development DPE-5542-G-SS-A042-00.

Kosovel, V., A. Avanzini*, V. Scarcia and A. Furlani. SEASONAL VARIATIONS OF THE CYTOSTATIC ACTIVITY IN DICTYOPTERIS MEMBRANACEA

Among the screened algae of the Gulf of Trieste for the cytostatic activity, particularly interesting appears to be the activity in all the three different crude extracts (boiling water, 30% ethanol, chloroform) obtained from the brown alga D. membranacea present in the Gulf of Trieste in all the seasons of the year.

The present work refers about the "in vitro" assays against KB cell growth, carried out at monthly intervals with D. membranacea crude extracts. The results indicate that the production of cytostatic substances by this alga is clearly affected by the seasons of the year, in fact the activity varies from very high values in the month of December (68;52;43% of growth inhibition at 0.1 mcg/ml of the culture medium in chloroform, ethanol and aqueous extract respectively) to no activity in the months from January to June during the year 1988.

Noda, H., H. Amano and K. Arashima and K. Nisizawa. ANTITUMOUR ACTIVITY OF MARINE ALGAE.

Antitumour effects of marine algae against in vivo screening (Ehrlich carcinoma and Meth A fibrosarcoma) and in vitro screening (human cancer cell lines) were studied from the view point of possible utilization for tumour preventing material. Of these results, particularly should it be pointed out the fact that several powder such as Sargassum riggoldianum, Laminaria angustata, Eucheuma cottinii and Porphyra yezoensis showed relatively high antitumour activities. Partially purified polysaccharides, i.e. fucoidan, carageenan and porphyran preparations from seaweed mentioned above were effective against both Ehrlich carcinoma and Meth A fibrosarcoma. The antitumour activities of neutral lipid, glycolipid and phospholipid fractions from S. ringgoldianum, and those of glycolipid and phospholipid fractions from L. angustata were of high values. Phospholipid fraction rom P. yezoensis showed a very high activity.

Hori, K.*, K. Miyazawa and K. Ito. SOME COMMON PROPERTY AMONG LECTINS FROM MARINE ALGAE.

It has now been demonstrated that hemagglutinins were widely distributed in marine algae. Nevertheless, agglutinins(or lectins) have been isolated and characterized only from several species. It is one goal of our study to elucidate the structure and function of lectins from marine algae, which will be useful for understanding of molecular evolution of lectins from lower to higher plant, and for evaluation of fine chemicals from marine algae.

We have to date isolated and characterized totally 12 lectins from 4 species of marine algae. These lectins revealed monomeric proteins or glycoproteins with the low-molecular weight of 4,200 to 25,000. Interestingly, a Hypnea japonica lectin, "Hypnin A", was a simple peptide consisted of 43 amino acid residues. The activities of these lectins were commonly inhibited by glycoproteins bearing N-glycosidic sugar chains, but not by any monosaccharides tested. Some common properties of marine algal lectins will be presented in camparison with lectins from higher plants.

V. Vreeland,* E. Zablackis, M. Gretz, N. Stanley, D. Stancioff, F. H. Kirkpatrick and W.M. Laetsch. ELECTROPHORETIC ANALYSIS OF CARRAGEENAN SUBSTRUCTURE AND INTERACTIONS.

Polyacrylamide electrophoresis was used to study the size distribution, purity and interactions of kappa, iota and lambda carrageenan fragments. The native size distribution of kappa and iota subunits was evident after treatment with iota- and kappa-carrageenases, respectively. Kappa and iota subunits had distinctive band patterns and color when silver stained. The multiple bands of each repeating pattern may represent differences in sulfation, anhydrogalactose content or chain length. The purity of kappa and iota samples was assessed by the presence of contaminating bands. No regular pattern was seen for lambda carrageenan after treatment with lambda-, kappa- or iota-carrageenases or after acidic hydrolysis. Calcium precipitated short kappa but not iota subunits. Relatively soft iota gels apparently form by dimerization only. Supported by California Sea Grant R/MP-36, Marine Colloids Division of FMC, Inc., and Agency for International Development DPE-5542-G-SS-A042-00.

Cosson, J., E. Deslandes and J.P. Braud * SEASONAL VARIATIONS IN CARRAGEENANS FROM FOUR CARRAGEENOPHYTES ON THE NORMANDY COAST (FRANCE).

Carrageenans, extracted by alkaline transformation, were studied by spectroscopic and physicochemical methods in some Rhodophyceae from Normandy coasts. Among them, four species which synthesized ι-carrageenan were studied during the year : Calliblepharis ciliata (Huds.) Kütz., C. jubata (Good. et Woodw.) Kütz., Cystoclonium purpureum (Huds.) Batt. and Gymnogongrus crenulatus (Turn.) J. Agardh. Carrageenan quantities changed with the seasons : maximum at the end of spring and minimum in autumn, in relation with growth and reproductive periods of this algae.

Chopin, T.*[1], Hanisak[2], M.D., Koehn[2], F., Mollion[1], J., and S. Moreau[3].
[1]EPPV, Univ. Lille, 59655 Villeneuve d'Ascq Cédex, France. [2]Harbor Branch Oceanographic Inst., Fort Pierce, Florida, 34946 U.S.A. [3]INSERM-U42, 59650 Villeneuve d'Ascq Cédex, France.
EFFECTS OF PHOSPHORUS CONCENTRATION IN SEAWATER ON GROWTH AND CARRAGEENAN CONTENT IN AGARDHIELLA SUBULATA (RHODOPHYCEAE, GIGARTINALES).

Infrared and ^{13}C-NMR spectroscopies, gas liquid chromatography, and chemical analysis showed that phycocolloids extracted from A. subulata had a dominant ι-carrageenan feature. Unattached plants were cultivated for 4 weeks in tanks receiving seawater enriched with 53.5 µM N and with 0 to 20 µM P/week. Maximal growth rate and carrageenan content were observed with an enrichment of 6 µM P and 3 µM P, respectively. Diminished results were obtained at either lower or higher P concentrations. Hence, carrageenan production was promoted in the range of 3-6 µM P. Further P enrichment was useless. This phenomenon, observed while studying P nutrition, compared with the Neish effect shown in N nutrition studies.

Deslandes, E.*, P. Potin and J.Y. Floc'h. CONTRIBUTION ON THE CONTENT AND NATURE OF THE PHYCOCOLLOID FROM KALLYMENIA RENIFORMIS (TURNER) J. AGARDH.

The red alga Kallymenia reniformis (Rhodophyta, Cryptonemiales) collected on the west coast of Brittany contained 43 % ash, 5.6 % crude protein and 38.7 % soluble carbohydrate. The phycocolloid extract (38 % dry weight) was investigated using chemical and spectroscopic (I.R. and ^{13}C N.M.R.) methods. Preliminary results show that this polysaccharide belongs to the lambda-carrageenan family.

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THE PRIMARY CELL WALL OF A NON-GRAMINACEOUS MONOCOT ASPARAGUS

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Angiosperms are generally categorized as either monocot or dicot. This classification scheme understates the differences between graminaceous and non-graminaceous monocots. We aim to utilize the primary cell wall as a tool for study of the relationship between the graminaceous monocots, the non-graminaceous monocots, and the dicots. Prom. :c digestion of HF-deglycosylated asparagus primary cell wall liberates a peptide whose composition suggests an intermediate relationship between the graminaceous monocot, maize, and the dicots. Amino acid analysis of this peptide indicates an empirical formula containing $SerHyp_2Pro_3$ together with Isodityrosine. Underhydroxylation of extensin occurs in maize. This evidence supports the close relationship between asparagus(a non-graminaceous monocot) and the graminaceous monocots. On the other hand, the presence of IDT indicates less divergence from the dicot primary cell wall. HPLC analysis cor. borates the amino acid analysis data suggesting the presence of IDT. In addition, GCMS analysis will provide sequence data for this peptide and confirm the presence or absence of IDT.

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Effect of Chitosan on Callose Synthase in Plant Cell Suspension Cells.

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Department of Food Science, University of Delaware, Newark, De

The effect of chitosan on a callose synthesizing enzyme was examined in carrot and Chenopodium suspension cells. Callose synthase was most active at the earlier stages of growth, when the cells were activity dividing. Chitosan stimulated callose synthase at days 2 and 10 in culture. However, chitosan had little effect on the isolated microsomal enzyme. Glucans synthesized were perdominately B-1,3 linkaged. The activity of chitinase and ATPase will also be discussed.

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PYROLYSIS-GC-MS CHARACTERIZATION OF PLANT CELL WALLS

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Pyrolysis has been shown to fragment plant materials into diagnostic components that are indicative of the parent molecule. We have used pyrolysis-GC-MS to analyze plant cell walls. Pyrolysis was carried out using a CDS pyroprobe set to ballistically heat 250-750 µg samples to 700°C and to carry the volatile components onto the top of 60m DB-1 capillary column. The GC temperature was held at 50°C to trap and focus volatile materials and then ramped to 275°C at 4°C/min. Compounds eluting from the column were identified by their mass spectra as lignin-related, carbohydrate-related, or extractives. We have used this technique to compare cell walls from different plant tissues. Lignin- and phenolic-derived fragments have diagnostic fragmentation pathways in mass spectrometry and provide a wealth of information. Carbohydrate-derived compounds are more difficult to interpret but do provide information on general classes of polysaccharides present.

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ELECTROPHORETIC ANALYSIS OF POLYGALACTURONATE, ALGINATE AND CARRAGEENAN SUBSTRUCTURE AND PROPERTIES

Valerie Vreeland,[1] Earl Zablackis,[2] Bogdan Doboszewski,[1] Michael Saxton,[3] and Watson M. Laetsch[1] Univ. Calif: [1]Plant Biol. Dept., Berkeley, CA 94720; [2]Biol. Sci. Dept., Santa Barbara, CA 93106; [3]Plant Growth Lab, Davis, CA 95616

Polyacrylamide electrophoresis was used to separate micrograms of polygalacturonate, alginate and carrageenan fragments into discrete bands, each band differing in length by one sugar unit. Carbohydrate from 1 mg of tissue was analyzed, resolving fragments 4-100 sugar units in length. Each carbohydrate fragment type had a distinctive band pattern. The native size distributions of gelling and nongelling subunits were determined after specific enzymatic cleavage. Ion fractionation of subunit types revealed new information on subunit interactions. An alginate subunit arrangement was identified with gelling subunits which could not gel due to kinks. Of the two carrageenan gelling subunits, kappa forms aggregates in calcium ions but iota cannot and presumably forms dimers. Microscale elution and purification of fluorescent subunits was used to prepare monodisperse probes to study gelling interactions.

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SIMULTANEOUS ANALYSIS OF OLIGOGALACTURONIC ACIDS WITH AND WITHOUT A 4,5-UNSATURATED FUNCTION AT THE NONREDUCING END

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Traditionally A_{235} detection of 4,5-unsaturated bonds has been used to evaluate the depolymerization of pectic polysaccharides by pectate lyase. This approach when used to monitor the changes in degree of polymerization (DP) ignores the generation of oligogalacturonic acids without 4,5-unsaturated function that appear early in the time course of pectate lyase cleavage. We have utilized high performance anion exchange chromatography (Dionex CarboPac PA1 column, pH 6 oxalate buffer nonlinear gr...: at mobile phase) coupled with pulsed amperometric detection (Anal. Biochem. 184 (1990) 200-206) in order to separate and detect oligogalacturonic acids both with and without 4,5-unsaturated functions at their nonreducing ends in pectate lyase digests. Preparative HPLC purified oligogalacturonic acid standards were used to identify peaks in pectate lyase digest chromatograms. Retention time comparisons were also made with a polygalacturonic acid autoclave hydrolysate that contained oligogalacturonic acids up to a DP of 50 that lacked the 4,5-unsaturated function. When comparing like DP oligogalacturonic acids, those without the 4,5-unsaturated funct. n eluted earlier from the CarboPac PA1 column than did their unsaturated counterparts. These techniques provide the first means for the DP assignment of oligogalacturonic acids generated by both lytic and hydrolytic cleavage of polygalacturonic acid and plant cell walls.

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LIGHT-INDUCED INHIBITION OF CELL DIVISION IN VITTARIA

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Gametophytes of the fern Vittaria graminifolia bear 6-celled propagules called gemmae. Four of the cells may divide asymmetrically, producing a smaller cell which differentiates into a rhizoid or prothallus. When gemmae are grown under continuous white light, division is promoted by increasing light intensity, up to a maximum at 2w/m2. Greater intensities inhibit division in a dose-dependent manner, by causing a delay before division begins. The maximal division rate, however, is unaltered. A 24 hour high light intensity pulse also delays division, even when given 1-3 days before division begins. Apparently, a phase preparatory to mitosis exists, during the G1 or S phase, which can be regulated by light intensity. The effect of high light intensity on division is not attributable to an inhibition of photosynthesis.

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Bamfield Marine Station, April 1991

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ALGINATE AND CARRAGEENAN SUBUNIT ANALYSIS WITH SPECIFIC MARKERS AND ELECTROPHORESIS. Valerie Vreeland. Department of Plant Biology. University of California, Berkeley, CA 94720. USA

Phycocolloids such as carrageenan and alginate are carbohydrates extracted from the cell walls of seaweeds. The same gelling, viscosity and binding properties responsible for their commercial value are important for algal wall structure and function. However, variability in polydispersity, composition and structural organization have limited both commercial development and understanding how wall structure is controlled. We have employed two approaches from nucleic acid and protein research to study the structure/function relationship of these phycocolloids. These are *in situ* localization of gelling and nongelling sequences with molecular markers (monoclonal antibodies and hybridization probes) and electrophoretic analysis of phycocolloid fragments.

Carrageenan is a family of sulfated galactan polymers having several types of disaccharide repeating units along hybrid molecules. Kappa carrageenan forms a firm gel, iota carrageenan formas a weak gel, and lambda carrageenan is highly sulfated but does not gel. We have studied kappa and iota carrageenan in red algae from seaweed farms in the Philippines. Plants having predominantly kappa carrageenan (*Kappaphycus alvarezii*) and iota carrageenan (*Eucheuma spinosum*) are propagated vegetatively. The quality of carrageenan harvested is variable, and a decrease in gel strength of farmed carrageenan possibly due to interbreeding has been observed with time. Monoclonal antibodies to kappa, iota, lambda, precursor and other carrageenan components were used in tissue blot and dot blot assays to rapidly compare carrageenan composition and purity in farmed plants and in carrageenan-containing food products. Tissue blotting clearly showed significant differences among farmed plants, and can contribute to selection of seedling plants which produce high quality carrageenan. Large medullary cells are generated from a peripheral meristematic cell layer in *Kappaphycus alvarezii*, and intracellular carrageenan synthesis was seen in young branches by immunofluorescence. Large amounts of low-gelling precursor carrageenan were found intracellularly in medullary cells of rapidly-growing plants, while less was seen in slowly-growing plants.

Electrophoretic analysis of carrageenan fragments was accomplished on polyacrylamide gels. Kappa and iota carrageenan each produced a complex and distinctive band pattern after partial cleavage by acid or specific enzymes (kappa carrageenase and iota carrageenase). However, no bands were seen for lambda carrageenan, which therefore has no regular repeating structure.

Alginate production was studied during cell wall assembly in zygotes of the brown alga, *Fucus*. Alginate, the major cell wall carbohydrate from brown seaweeds, is a linear polymer of two sugar units arranged into three types of sequences of variable length and with different properties. Polymannuronate and alternating mannuronate-guluronate segments are nongelling, while polyguluronate segments form gels in the presence of calcium. Ultrastrucural immunogold labeling of zygotes with monoclonal

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antibodies specific for the gelling subunit revealed that their biosynthesis occurs in Golgi vesicles. The intracellular addition of gelling subunits to alginate in this alga differs from the extracellular addition in the bacterium *Azotobacter vinelandii*. Alginase activity was found associated with localized wall-loosening during germination of zygotes. The termini of gelling segments were labeled in the mucilage surrounding the rhizoid produced at germination, while gelling segments were labeled in the fibrillar rhizoid wall. This provided evidence for both a gelling function for polyguluronate in wall fibrils and a nongelling function in the mucilage which mediates adhesion of the zygote to the substrate. Phenolic compounds were also localized to the mucilage. These observations led to a hypothesis for the mechanism of adhesion in brown algal zygotes: phenolic materials crosslink carbohydrate fibers and serve as adhesion sites, in a manner analogous to the adhesion mechanism of marine invertebrates such as mussels.

Alginate subunits and polygalacturonate were separated by electrophoresis on acrylamide gels. Each band represented a discrete chain length and each subunit type had a characteristic band spacing. Migration of oligouronates ten sugars in length showed a pattern reversal in the presence or absence of calcium ions. Polyguluronate has a buckled shape and a higher charge density, so it migrates the most rapidly in an electric field. However, it binds calcium ions tightly and migrates the slowest in the presence of calcium ions. This ion electrophoresis demonstrates differential calcium binding for alternating sequences in addition to polyguluronate. Similarly, when acid-cleaved alginate fractions are precipitated with divalent cations at low ionic strength, all but the shortest alternating fragments precipitate. This demonstrates ion-mediated aggregation of this alginate subunit previously thought to be totally nongelling. Electrophoretic analysis was used to follow the time course of enzymatic degradation of alginate, and to examine the nature of the products. Mannuronase and guluronase cleavage was used to determine the size distribution of polymannuronate and polyguluronate sequences in alginates, since the size of these nongelling and gelling subunits is important to the structure/function relationship for alginates.

A very interesting finding resulted from combining ion fractionation with enzymatic cleavage of alternating sequences. All but the shortest alternating sequences were precipitated by calcium ions while smeared material remained calcium-soluble. A polyguluronate band pattern was revealed in this material by mannuronase while complete degradation was obtained with guluronase, due to one or a few mannuronate units apparently interrupting polyguluronate aggregation. This unexpected result revealed that a significant proportion of gelling subunits are functionally nongelling due to their structural organization. This nongelling type of alginate sequence organization may correspond to the nongelling alginate found in the zygote mucilage.

In conclusion, phycocolliod subunit analysis by electrophoresis and localization is providing information on the carbohydrate structure/function relationship which was previously diffi-

cleaved alginate or carrageenan revealed subunit size and type on small amounts of multiple samples. A nongelling arrangement of alginate gelling subunits was found which ,ay be associated with an embryo adhesion function. Carrageenan composition of individual plants and product mixtures was rapidly determined using specific monoclonal antibodies on tissue and dot blots.

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In: Tissue Printing, Tools for the study of Anatomy, Histochemistry and Gene Expression. eds. R. Pont-Lezica, P. Reid, E. del Campillo and R. Taylor. Academic Press (In press).

Immunolocalization of carrageenan components in seaweeds.

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Carrageenans, a family of variable sulfated galactans, are the major wall component in some red algae. These linear polymers are based on a D-galactose disaccharide repeat with alternating _-1,3 and ß-1,4 linkages. Sulfation sites and anhydrogalactose content differ for kappa, iota, lambda and other carrageenan types. Kappa forms firm gel gels, iota forms soft gels and lambda is nongelling.

Kappa- and iota-carrageenophytes farmed in the Philippines are commercial sources of carrageenan for food and other industrial applications. The farmed plants reproduce vegetatively (Azanza-Corrales and Dawes 1989). They also sporulate to produce wild plants (Doty 1985, 1987; Azanza-Corrales 1990), including possible hybrids which could contaminate commercial stock and cause the decrease in carrageenan gel strength recently observed (Azanza-Corrales 1990) when included with farmed plants by subsistence farmers. Tissue printing can be used to screen potential seed stock on reef platforms in 1-2 hours. Carrageenans bind to quaternary amino groups on positively charged nylon membranes for immunolabeling of tissue prints. We prepared monoclonal antibodies as molecular markers for carrageenan types, and immunofluorescence showed differential extracellular localization of carrageenan antigens in Kappaphycus (Vreeland et al. 1988; Vreeland et al. submitted). The anti-carrageenan monoclonal antibodies are mostly IgM, like many anti-carbohydrate antibodies. Second antibodies to the light chain of the monoclonal antibody (a kappa light chain in most cases) gives much stronger labeling than anti-mu heavy chain (V. Vreeland and X. L. Wang, in preparation).

Tissue printing of farmed kappa- and iota-carrageenophytes followed by labeling with monoclonal antibodies to carrageenan clearly differentiates the two kinds of commercial stock. Tissue printing is being used to identify individual plants with pure or mixed carrageenan types.

1. Materials

1. Biodyne B nylon membrane (Pall BNAZF3R), 0.2 µM pore size.
2. 1% ovalbumin (Sigma grade 2) in 50 mM Tris, pH 7.5 and 0.12% sodium azide. Centrifuge at 12,000 g and store aliquoted at -20°C.

3. Wash solution: 200 mM NaCl and 10 mM $CaCl_2$ adjusted to pH 7.0 with NaOH.
4. Anti-carrageenan monoclonal antibodies in hybridoma culture medium (Vreeland et al. 1988, and Vreeland et al. submitted). Hybridoma medium contains Iscove's Modified Dulbecco's Eagles Medium with 20% fetal or newborn bovine serum.
5. Alkaline phosphatase-conjugated goat anti-mouse antibody (kappa chain specific; Southern Biotechnology, distributed by Fischer) is used as the second antibody.
6. Alkaline phosphatase substrates: 5-bromo-4-chloro-3-indoyl phosphate, p-toluidine salt (BCIP) and nitroblue tetrazolium, (NBT) grade 3 (Sigma). BCIP stock solution: 4 mg/ml BCIP in 1:2 acetone:methanol, stored at -20°C. Note: if a precipitate forms in the stock solution, it can be dissolved by briefly warming. If its activity is low or a dark bluish background results, the precipitated stock should be discarded.
7. Substrate buffer: 200 mM Tris and 4 mM $MgCl_2$ at pH 9.5. (24.2 g Trizma base + 810 mg $McCl_2 \cdot H_20$, diluted to 1 l with distilled water.)
8. Substrate solution: 0.75 ml of BCIP stock solution and 5 mg of NBT are added to 50 ml of substrate buffer immediately before use. The substrate solution can be stored in the dark, preferably in the refrigerator, for re-use on the same day. It should be discarded when a blue/purple precipitate forms.
.lm1

2. Plant material

Kappaphycus alvarezii = Eucheuma alvarezii Doty (produces kappa carrageenan; commercial name is E. cottonii) and Eucheuma denticulatum (Burman) Collins et Hervey (produces iota carrageenan; commercial name is E. spinosum) from commercial seaweed farms in the Philippines. Fresh, frozen, dried or formalin-fixed plants may be used for printing. Fresh plants give the sharpest prints. Dried plants are washed briefly in tap water, and then rehydrated for 30 min in 500 mM KCl + 50 mM $CaCl_2$ prior to printing.

3. Procedure

1. Cut Biotrans B membrane with scissors to the minimum necessary size. Identify membrane by pencil notation in a corner. Use fine forceps and vinyl gloves to handle membranes. Place a piece of whatman #1 filter paper under the membrane.
2. Cut tissue on a polyethylene or other stiff plastic sheet 1-2 mm thick with a clean, sharp single-edge razor blade. Transfer tissue to the membrane with fine forceps, taking care not to move the tissue after first contact with the membrane. Finger-press the tissue firmly for 30-60 seconds (with a clean glove), and then remove the tissue with forceps. Air-dry the print.

3. Block the membrane by incubation for 15 minutes in ovalbumin with gentle agitation on a rotary shaker. Rinse briefly in wash solution. Blot excess liquid from the edge of the membrane onto paper towel.
4. Place the damp membrane (print side up) on a dry plastic surface in a small covered container to prevent evaporation. A 6-well tissue culture plate is convenient for multiple antibody incubations. Incubate the membrane in a minimal volume (25-50 $\mu l/cm^2$) of monoclonal antibody for 15 min. The hybridoma supernatant is transferred with a sterile, disposable pipet tip to avoid contamination of the antibody supply.
5. Wash the membrane briefly and then transfer it to a larger volume of fresh wash solution. Gently agitate it for 4-5 minutes. Remove excess liquid from the membrane.
6. Dilute the second antibody 1,000-fold into hybridoma medium. Cover the damp membrane with diluted second antibody and incubate 15 min.
7. Wash as in step 5.
8. Several membranes are incubated in the substrate solution for 5-30 minutes with gentle agitation until the background darkens.
9. Stop color development by a brief wash in distilled water. The results should not be analyzed until the print is dry, since the background lightens upon drying and wall impressions appear dark until completely dry.
10. Photograph BCIP/NBT-stained tissue prints on Technical Pan film (#2415, Estar Base, Kodak) at ASA 125-160 and develop in HC-110 developer (Kodak), dilution B for 8.5 min at 22-23° C for high contrast.
.lm1

Figure Legends

1. Comparison of cross sections of a brown kappa-producing plant (a,d), a brown iota-producing plant (b,e) and a brownish-green apparent hybrid plant (c,f) with two anti-carrageenan antibodies, 26-3G1-1C4 to lambda carrageenan (a-c) and 26-6A11-2A4 to kappa carrageenan (d-f). The hybrid plant had the branching morphology of a kappa-producing plant but also had a few short spines on the main axis, similar to the numerous spines on iota-producing plants. Plants were collected at the FMC seaweed farm at Tindog beach, Cebu, the Philippines, with identification by Rubin Barraca of FMC. Prints were made from fresh plants on the day of harvest, air-dried and labeled several weeks later in Berkeley. Scale bar is 1 mm.

2. Antibody 26-6A11-2A4 to kappa carrageenan labels intracellular carrageenan (arrowheads) inside some large (0.2-1.0 mm diameter) medullary cells in a formaldehyde-fixed, large-diameter green kappa-producing plant from Tindog beach. Scale bar is 1 mm. Immunogold ultrastructural localization had revealed intracellular synthesis of carrageenan components on embedded sections of a red alga (Gretz et al. 1990)

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Figure
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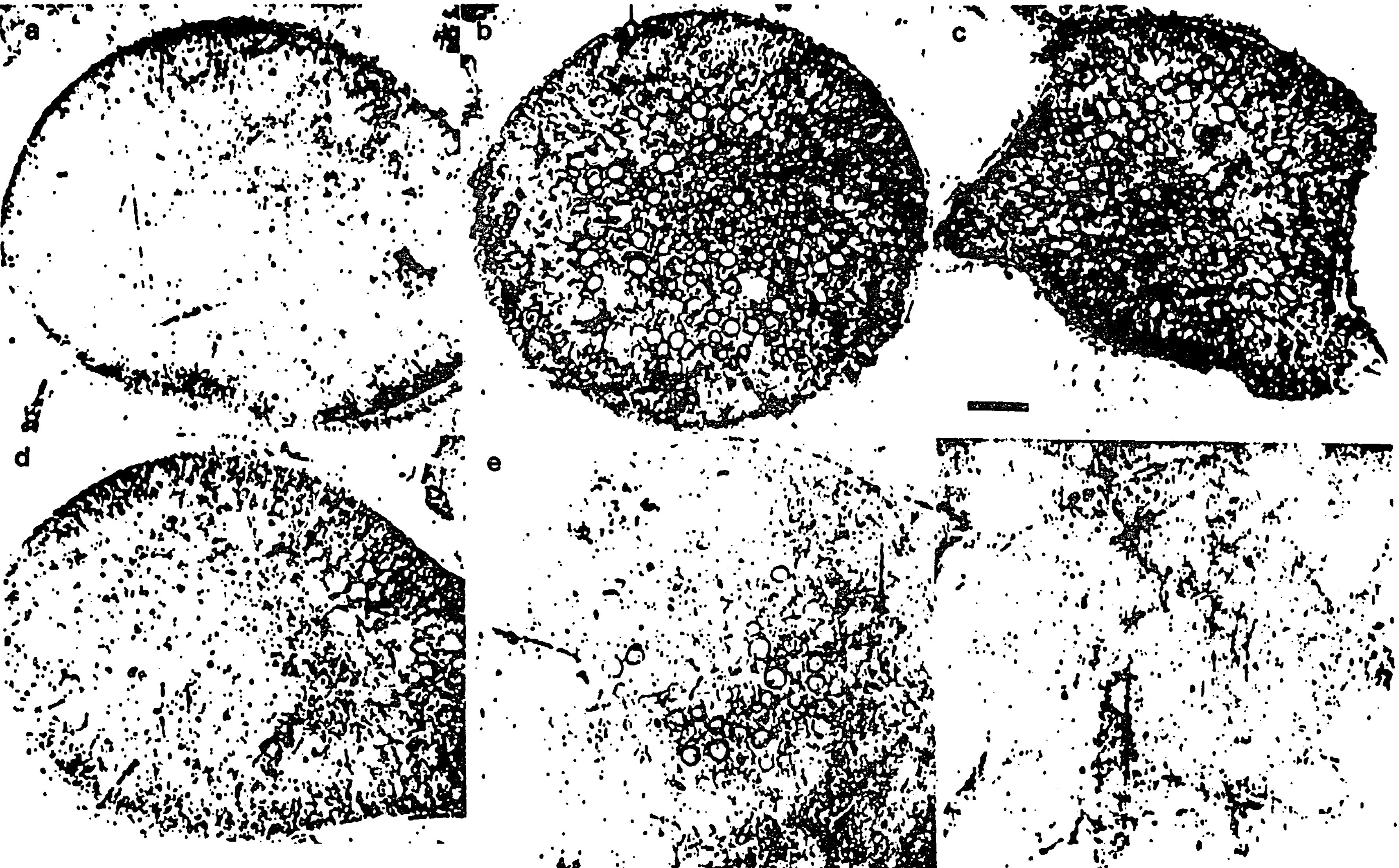
Figure 1



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MONOCLONAL ANTIBODIES AS MOLECULAR MARKERS FOR THE INTRACELLULAR AND CELL WALL DISTRIBUTION OF CARRAGEENAN EPITOPES IN *KAPPAPHYCUS* (RHODOPHYTA) DURING TISSUE DEVELOPMENT[1]

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ABSTRACT

Carrageenan, the major cell wall carbohydrate of certain red algae, is variable in structure and gelling properties. Sequence types include gelling (kappa and iota) and nongelling (lambda) types in addition to precursors, often in hybrid molecules containing more than one precursor and/or sequence type. Molecular markers to subunits were needed to study carrageenan synthesis, cell wall organization, and the relationship between structure and function. Monoclonal antibodies were produced to carrageenan, and their specificities were determined by competitive enzyme immunoassay. Antibodies were identified with specificities related to kappa, iota, and lambda carrageenan. The patterns of immunofluorescence localization on Kappaphycus alvarezii = Eucheuma alvarezii *var.* tambalang *(Doty) sections were distinctive for each antibody. The antibody to a kappa-related epitope labeled mature tissue strongly; antibodies to an iota-related epitope and a lambda-related epitope labeled weakly, consistent with the kappa-enriched carrageenan produced by this alga. Kappa-related epitopes were distributed throughout the wall and matrix, whereas iota-related epitopes were concentrated in the middle lamella. Lambda-related epitopes were localized primarily at the plant cuticle where kappa and iota antigens were lacking. An antibody appeared to be specific for a precursor of the gelling subunits because it showed maximal wall and intracellular labeling at the youngest developmental stage. All antibodies labeled intracellular inclusions in the transition zone between the epidermis and medulla during the development of medullary cells from the peripheral meristem in young branches. The results demonstrate the intracellular synthesis of epitopes related to all major carrageenan subunits and their differential extracellular distribution.*

Key index words: cell wall; enzyme immunoassay; Eucheuma alvarezii *var.* tambalang; *immunofluorescence; iota carrageenan; kappa carrageenan;* Kappaphycus alvarezii; *lambda carrageenan; localization; monoclonal antibodies; Rhodophyta*

Little is known about the distribution of different types of carrageenan within a red algal thallus, and less is known about biosynthesis and modification of carrageenan molecules during growth and development of algae. The *in situ* synthesis and distribution of carrageenan types was investigated in *Kappaphycus alvarezii* Doty = *Eucheuma alvarezii* var. *tambalang* Doty (Doty 1988), a major commercial source for kappa carrageenan (Santos 1989). Antisera to carrageenans played an instrumental role in the discovery of the biochemical alternation of carrageenan type associated with ploidy level in the Gigartinaceae and Phyllophoraceae (Gordon-Mills and McCandless 1975, McCandless et al. 1981). Molecular markers such as polyclonal antibodies (Vreeland 1970, 1972, Gordon-Mills and McCandless 1975, DiNinno and McCandless 1978), monoclonal antibodies (Vreeland et al. 1984, 1987, 1988, 1989, Larsen et al. 1985, Vreeland and Laetsch 1985, 1988), and hybridization probes (Vreeland et al. 1987, Zablackis et al. 1988, 1991, Vreeland and Laetsch 1989) have been prepared for marine algal cell wall carbohydrate subunits.

The processes of sexual and vegetative reproduction in *Kappaphycus* and *Eucheuma* are poorly understood. During rapid vegetative growth of farmed *Kappaphycus alvarezii* var. *tambalang* (Doty 1985), thallus mass increases by tip elongation, thickening of the roughly cylindrical thallus, and branching. However, the anatomical details of *Kappaphycus* remain largely undefined (Doty 1987). Regeneration of thallus cuttings (Azanza-Corrales and Dawes 1989) is the basis of commercial *Kappaphycus* growth. Details of sexual reproduction are poorly understood in *Kappaphycus* (Azanza-Corrales 1990), although diploid sporelings have been reported (Doty 1987) and very small plants are found attached to substrata in the native habitat (Doty 1985). *Kappaphycus* contains three types of tissues (Doty 1985, Doty and Norris 1985). Vegetative morphology and production of cell files in an iota carrageenan-producing *Eucheuma* species was described by Gabrielson (1983). Radially elongate cortical or epidermal cells at the thallus surface surround a medulla of enlarged spherical cells, and, in younger branches (up to a few centimeters in length), a medullary core of smaller-diameter cells is present. Thallus morphology and tissue organization vary and can change in appearance during preservation (Doty 1988).

The backbone of carrageenan consists of a disaccharide repeat of D-galactose units having alternating 1,3- and 1,4-linkages. Major sequence types include three types of repeating units (Bellion et al. 1982, Greer and Yaphe 1984), which vary in sulfation site and sulfation content as well as in the presence of 3,6-anhydrogalactose (Fig. 1). Kappa

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carrageenan has a 4-sulfate on the 1,3-linked galactose, and the 1,4-linked sugar is anhydrogalactose. Iota is similar to kappa but also contains a 2-sulfate on the anhydrogalactose unit. Lambda lacks anhydrogalactose and contains sulfate at up to three sites. The precursor to kappa carrageenan is mu carrageenan, in which the 3,6-anhydrogalactose unit is replaced by a galactose 6-sulfate unit. Carrageenan gelation is an important aspect of cell wall structure, and the gelling properties of carrageenan are related to composition of disaccharide types. Low sulfate and high 3,6-anhydrogalactose are correlated with high gel strengths (Glicksman 1983). Both kappa and iota sequences contain anhydrogalactose in the disaccharide repeat and form gels; kappa gelation occurs optimally with potassium ions (as measured by gel strength) and forms a stiff gel, whereas optimum iota carrageenan gelation occurs in the presence of calcium and magnesium ions, forming soft elastic gels (Smidsrød and Grasdalen 1984). Lambda and 6-sulfated precursor carrageenans lack anhydrogalactose and do not form gels. Some precursor disaccharides remain in kappa and iota molecules and can be converted to anhydrogalactose by alkaline treatment, with a concomitant increase in gel strength (Glicksman 1983).

Carrageenan is highly variable in structure, and preparations are not homogeneous. For example, so-called kappa or iota carrageenans usually contain kappa/iota hybrid molecules (Bellion et al. 1982). Precursor units and other sequences and modifications can also be present in the carrageenan molecules. This heterogeneity has caused difficulties in studies of carrageenan molecular organization, biosynthesis, and *in situ* distribution. It is also a major consideration in evaluating possible carrageenan structures or epitopes recognized by antibodies. Antibody recognition is based on antigen shape and charge properties. The uncharged anhydrogalactose unit and resulting helical shape may be involved in epitopes for the gelling subunits (kappa and iota). In addition, the 4-sulfate of kappa and iota and/or the 2-sulfate of iota could be involved, as could the 6-sulfates on lambda and precursors. Sulfate groups are negatively charged and bulky. Sulfates at specific sites could either aid or hinder antibody binding, depending on the epitope. Epitopes can be up to seven sugars in length and can contain repeats of more than one type. For example, an antibody could recognize one kappa disaccharide plus an iota disaccharide, or a kappa disaccharide plus a precursor disaccharide in a certain sequence.

In this study, monoclonal antibodies were prepared to carrageenan, characterized by competitive immunoassay, and employed as molecular markers for evaluation of carrageenan epitope distribution and changes during wall formation in vegetative reproduction of *Kappaphycus*. A preliminary account of part of this work has been published (Vreeland et al. 1988). The vegetative tip was examined over a range of diameters, corresponding to increased tissue age and development with distance from the tip. The largest diameter studied was 4.6 mm, although thallus diameters of 2.5 cm occur commonly in older tissue (Doty 1985).

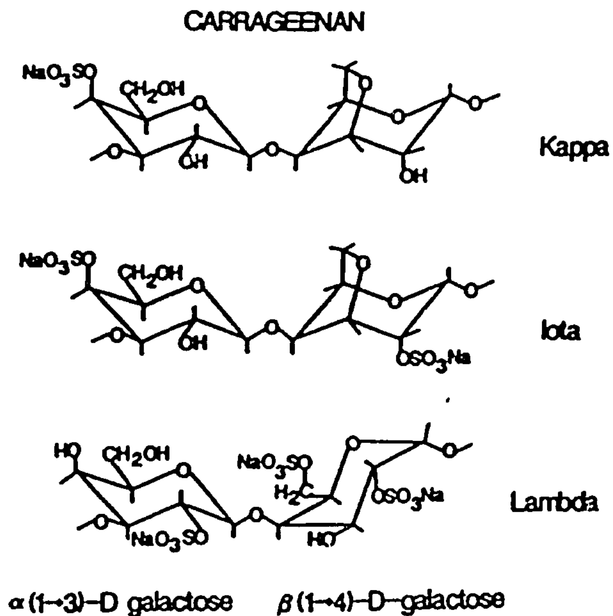

FIG. 1. The theoretical repeating structures of kappa, iota, and lambda carrageenan disaccharides differ in sulfation sites and content. The anhydrogalactose in kappa and iota is associated with gel formation.

MATERIALS AND METHODS

Carrageenan samples. Alkali-modified kappa (B375) and iota (B6700) carrageenans and lambda carrageenans (E389759 and from diploid *Chondrus crispus* lacking reproductive structures) were obtained from the Marine Colloids Division of FMC, Inc. (Rockland, Maine 04841-2996). Enzymatically prepared carrageenan oligosaccharides (β-neocarrabiose, neocarrabiose 4¹-sulfate, neocarratetraose 4¹,4³-disulfate, and neocarratetraose 4¹-sulfate) were purchased from Sigma (St. Louis, Missouri). Kappa and iota carrageenans were precipitated in 335 mM KCl to separate them from lambda carrageenan.

Short-chain kappa and iota carrageenans were prepared by mild acid hydrolysis of the high molecular weight commercial carrageenans from Marine Colloids. Carrageenan was dissolved in deionized water (0.67% w/v), adjusted to pH 2 with 150 mM HCl, and heated for 30 min in an 30° C water bath. Fragments were neutralized to pH 7 and recovered by precipitation in 2 volumes of 85% isopropanol, washed with 95% isopropanol, and dried overnight at 60° C. The short-chain kappa and iota carrageenan preparations were designated "short kappa" and "short iota," respectively, and the high molecular weight commercial kappa and iota carrageenan starting materials were designated "long kappa" and "long iota," respectively.

Carbon, nitrogen, and sulfate contents of carrageenan samples were determined by the Microchemical Analysis Laboratory (College of Chemistry, University of California, Berkeley) to determine the level of sulfation and the presence of protein. Sulfate and nitrogen contents were calculated relative to carbon content of carrageenan. No nitrogen was found in the kappa or iota carrageenans. Protein content (calculated as 15% nitrogen) was 2% for the crude long lambda carrageenan and 9% for the diploid *Chondrus* lambda carrageenan.

TABLE 1. *Sulfate and anhydrogalactose content of high molecular weight (long) and acid-fragmented (short) carrageenan samples. Theoretical values (ideal) and measured values calculated as disaccharide content.*

	Sulfate		Anhydrogalactose	
	Ideal	Measured	Ideal	Measured
Long kappa	1	1.14	1	0.97
Short kappa	1	0.8	1	0.90
Long iota	2	1.7	1	0.70
Short iota	2	1.6	1	0.73
Short lambda	3	1.8	0	0.22*
Diploid lambda	3	2.2	0	0.02

* This measurement is for long lambda.

Anhydrogalactose content was estimated according to the method of Yaphe and Arsenault (1965) with correction for the presence of 10% water. Sulfate and anhydrogalactose contents of the carrageenan samples are given in Table 1. The kappa contained more sulfate than expected. If this sulfate were due to iota subunits, the long kappa would contain about 15% iota subunits. Pyruvate content was estimated according to the method of Duckworth and Yaphe (1970) because pyruvate has been found in some carrageenans. No pyruvate was detected in the KCl-precipitated long kappa carrageenan sample, which was used as the standard solid-phase antigen in the enzyme immunoassay.

Infrared spectroscopy of the high molecular weight kappa and iota samples confirmed the predominant subunit type of these samples and revealed a minor 2-sulfate peak due to iota subunits in the kappa preparation (Zablackis et al. 1988). This iota contaminant was also seen by electrophoresis after kappa carrageenase treatment (Vreeland and Zablackis, unpubl.). Infrared spectroscopy was carried out according to the method of Santos and Doty (1975). The spectra of partially hydrolyzed kappa and iota carrageenans showed typical peaks at 1240, 930, and 845 cm^{-1} representing the S-O stretching vibration, the 3,6-anhydrogalactose, and the 4-sulfate of the 1,3-linked galactose unit, respectively. The iota spectrum had an additional peak at 805 cm^{-1} representing the 2-sulfate of the 3,6-anhydrogalactose residue.

Hybridoma preparation. Three strains of mice were immunized with carrageenan due to the variable and limited immunoresponsiveness of mice to carbohydrate antigens. Immunization was carried out at the Naval Biosciences Laboratory (Oakland, California) with the advice and assistance of Dr. Alexander Karu. The mouse stocks used in this study are now maintained by Dr. Karu at the College of Natural Resources Hybridoma Facility (University of California, Berkeley). Two mice each of Balb/C, B10/Q, and Biozzi high-immunoresponse (Biozzi et al. 1979) strains were immunized intradermally at multiple sites on the back with about 100 μg of carrageenan. The immunizing antigen for the first three doses was 2 mg·mL⁻¹ of short kappa carrageenan in 50 mM KCl, mixed with an equal volume of aqueous methylated-BSA (Sigma). MPL+TDM emulsion (Ribi Immunochem Research, Inc., Hamilton, Montana) was used as adjuvant. The Ribi adjuvant was reconstituted in half the recommended volume and mixed with an equal volume of the carrageenan solution to form an emulsion. For the fourth and fifth doses during the second month, long kappa and short kappa were used in equal proportions. A final booster was given 4 days prior to fusion, 6 months after initiation of immunization. The mouse used for Fusion 26, which produced all of the antibodies described in this paper, was a female Biozzi, 10 months old.

Monoclonal antibodies were prepared by a modification of the method of Vreeland et al. (1984) for antibodies to alginate. Mouse sera were screened for hybridomas producing anti-carrageenan antibodies by indirect immunofluorescence of cell walls in methacrylate sections of *Kappaphycus*. The myeloma parent cell line was P3X63-AG8.653. Macrophage-conditioned medium from the J774A.1 macrophage strain (Sugasawara et al. 1985) was used to support the growth of isolated hybridoma cells immediately after fusion and after cloning by limiting dilution so that less than 10% of the wells had cell growth. About five dozen cultures (6% of total cultures) from Fusion 26 with spleen cells from a single mouse were positive for carrageenan during preliminary screening by enzyme immunoassay. Eighteen hybridomas were selected by their strong indirect immunofluorescent labeling on cell walls. Eight of these hybridomas (Table 2) were successfully cultured. They were class-typed by immunofluorescence with fluorescein-conjugated anti-subclass antibodies (Southern Biotechnology Associates, Birmingham, Alabama) on the surface of hybridoma cells. All antibodies were IgM with kappa light chains, except antibody 3G1, which appears to be IgG, with a kappa light chain.

Tissue preparation. Kappaphycus alvarezii was grown in the laboratory under a 12:12 h LD photoperiod in ¼ strength modified PES medium (no vitamins or Tris) at 25° C. Thallus pieces were removed from culture and fixed in 2% formaldehyde and 2% glutaraldehyde in seawater for 1 h at 4° C. The pieces were cut into <1-mm-thick sections and fixed for an additional 12 h at 4° C. Fixative was removed by two 20-min rinses in seawater at 4° C. Tissue was postfixed in 2% osmium tetroxide in seawater at 4° C overnight and washed twice for 20 min each in seawater. Dehydration was carried out at room temperature in a seawater to 50% ethanol series and then in a 50% ethanol with deionized water to 100% ethanol series, to prevent salt precipitation. Each dehydration step was 1 h. Tissue was infiltrated with LR White soft resin or LKB JB-4 resin in 10% steps for 3 h each and polymerized at 60° C overnight. Sections were cut 2–4 μm thick on a Sorvall JB-4 microtome.

Retention of soluble carbohydrates was increased by postfixing sections with the photoactive fixative 1,3,5-triazido-2,4,6-trinitrobenzene (TTB; Polysciences, Warrington, Pennsylvania) according to Vreeland et al. (1989). Sections were treated with 5 mg·mL⁻¹ TTB in methanol for 15 min under UV light, rinsed with methanol, and air-dried. Sections were then incubated for 30 min in 50 mM EDTA at ambient temperature and rinsed twice with 50 mM EDTA prior to incubation with 200 mM KCl or 100 mM NaCl for 30 min and rinsing with the same salt solution.

Immunofluorescence. Mouse sera were screened for antibodies that labeled cell walls on sections of *Kappaphycus*. Hybridoma culture medium containing a monoclonal antibody was incubated on the pretreated sections for 15 min and sequentially rinsed with the same salt solution and the culture medium. Sections were incubated with mouse serum or mouse serum diluted 1:100 with medium for 60 min. Sections were rinsed and incubated with goat anti-mouse immunoglobulin (light and heavy chains) antibody (Antibodies Incorporated, Davis, California) diluted 1:100 in medium for 30 min and rinsed. Sections were covered with the antioxidant *p*-phenylenediamine (PPD; 25 mg dissolved in 2.5 mL 50 mM Tris buffer pH 8.6 and mixed with 22.5 mL glycerol) according to Vreeland et al. (1984). Sera from mice immunized for 1 and 2 months were tested for immunofluorescent labeling of cell walls in *K. alvarezii* sections. Preimmunization sera did not label cell walls. After the first month, the Biozzi and B10/Q antisera labeled the walls weakly. After the second month of immunization, sera from the two Biozzi mice labeled walls strongly. The B10/Q sera labeled moderately and the Balb/C sera labeled weakly.

Immunofluorescent labeling with cloned antibodies was modified from the preceding procedure for mouse sera. *Kappaphycus alvarezii* sections were not prewashed with EDTA, salt, or hybridoma medium. Twenty microliters of the monoclonal antibody in hybridoma medium were incubated on sections for 20 min. Sections were washed briefly in 150 mM NaCl plus 5 mM $CaCl_2$ at pH 7, and the second antibody incubation was 15 min. After washing, sections were mounted in Mowiol mountant (Heimer and Taylor 1974) containing the antioxidant, *n*-propyl gal-

late (Giloh and Sedat 1982). Antibody clones used for immunofluorescence labeling were 3G1-1C4, 4D12-1A12, 6A11-2A4, 7C2-3D5, 7E4-3E9, and 7H6-3D12. Antibody 5H12 was not available for labeling due to extremely slow growth of the hybridoma clone at that time.

Enzyme immunoassay. Initial screening of hybridoma supernatants was carried out by enzyme immunoassay as described by Larsen et al. (1985). The method was tested prior to anti-carrageenan antibody production with anti-agar monoclonal antibodies (Vreeland and Laetsch 1985), which cross-reacted with carrageenan in this assay. Long kappa carrageenan was coated directly on the microtiter plate. A monoclonal antibody was incubated on the plate and detected by goat anti-mouse immunoglobulin (gamma and light chain specific), conjugated to alkaline phosphatase (affinity purified; Tago, Burlingame, California). The substrate for alkaline phosphatase, disodium *p*-nitrophenyl phosphate (Sigma 104 alkaline phosphatase substrate tablets; Sigma Chemical Co., St. Louis, Missouri), was dissolved in 1 M diethanolamine-HCl buffer, pH 9.8, containing 0.05 mM $MgCl_2$ and 0.02% sodium azide. The reaction was stopped by addition of 100 µL of 100 mM L-cysteine in water (Brauner and Fridlender 1981).

Antibody specificity was studied with a competitive immunoassay modified from the preceding screening procedure:

Preparation of Plates: Casein, a milk protein that binds carrageenan (Hansen 1968, Towler 1975), was first coated on microtiter plates to increase carrageenan binding. This protein precoating step also eliminated the need to block remaining protein binding sites on the plate. Casein was dissolved by addition of KOH to pH 11–12 with stirring, and then HCl was slowly added to pH 8–9. A 1% casein stock solution was stored at −20° C. Immulon 2 microtiter plates (Dynatech Laboratories, Alexandria, Virginia) were coated with a 0.5% solution of casein (Calbiochem-Behring Corporation, La Jolla, California). Enzyme immunoassay reagents were added (100 µL per well) and washed (200 µL per well) in 50 mM KCl plus 5 mM $CaCl_2$ at pH 7 (salt solution). All incubations were at ambient temperature for 1 h. Aqueous 1 $mg \cdot mL^{-1}$ carrageenan stock solutions were stored at −20° C and diluted in salt solution immediately before use. Long kappa was the standard solid-phase antigen in order to eliminate any variation due to differences in binding by carrageenan types and sizes to casein. Casein-precoated plates were washed three times and incubated with 20 $\mu g \cdot mL^{-1}$ of long kappa carrageenan for either 1 h at ambient temperature or for up to 1 week at 4° C. Plates were then washed three times with salt solution.

Antibody Competition: A monoclonal antibody, in culture medium from an overgrown hybridoma culture, was diluted into the salt solution. The appropriate antibody dilution was determined in preliminary experiments as the highest dilution giving maximal O.D. response. The antibody was incubated with a soluble carrageenan sample, and then the monoclonal antibody–carrageenan solution was incubated on the carrageenan-coated plate. The plate was then washed three times with salt solution and incubated with a 1:1000 dilution of alkaline phosphatase-conjugated second antibody. It was necessary to dilute the second antibody in the hybridoma medium (Iscove's modified Dulbecco's medium with L-glutamine and 25 mM HEPES buffer (GIBCO Laboratories, Grand Island, New York) containing 20% newborn calf serum (Irvine Scientific, Santa Ana, California) to prevent high background with some antibodies. The plate was washed three times and incubated in substrate. The reaction was stopped by L-cysteine as earlier. The routine negative control was omission of the hybridoma antibody, and the positive control was the hybridoma antibody with no competitor. Enzyme immunoassay controls lacking carrageenan or with unrelated IgM were negative. The monoclonal antibodies served as additional, internal controls due to their different specificities.

Efficacy of competition by soluble carrageenan samples was tested by serial dilution. Competitor carrageenan samples were the high molecular weight commercial samples without further

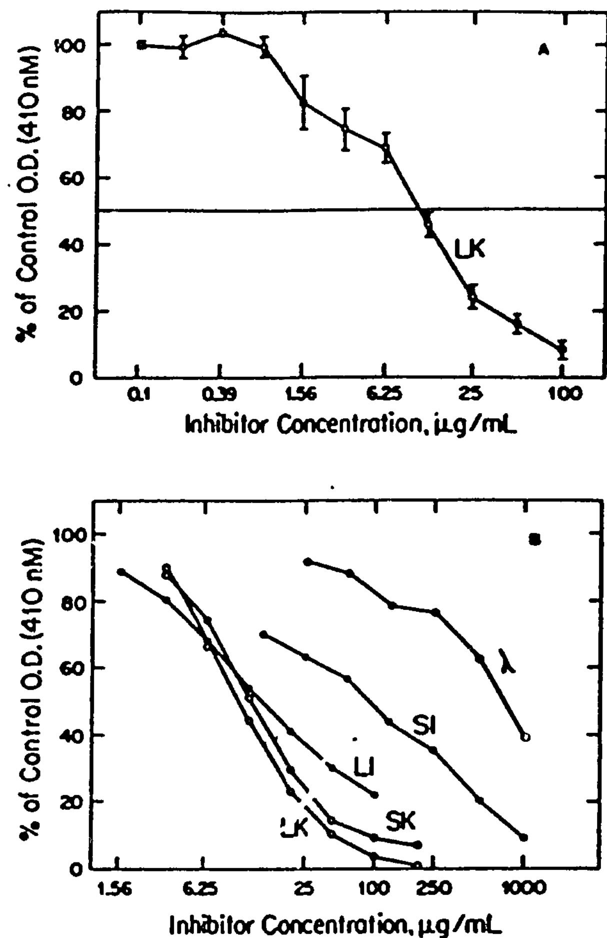

FIG. 2. Typical enzyme immunoassay competition curves. A) Inhibition of antibody 6A11-2A4 by long-chain kappa carrageenan, showing standard deviations for quadruplicate samples. B) Comparative inhibition of antibody 10A5 by long- and short-chain kappa and iota carrageenan and by lambda carrageenan.

purification and their partially acid-hydrolyzed short fragments. Four or six competition curves with constant antibody concentration and two- or threefold competitor dilution series were compared in a typical experiment. Long kappa carrageenan was the standard competitor for comparative purposes. Competition was compared by calculating the amount of each sample needed to reduce the positive control reaction by 50%. All data points were obtained from the average of quadruplicate wells. In a typical experiment, the positive control was about 1.4–1.6 $O.D._{410}$ units and the background was about 0.10–0.15 units. Calculation of standard deviations, antigen quantities for 50% competition, and graphing of data were carried out on computerized worksheets written by Carl V. Hanson. A competition curve showing typical standard deviations of four replicates is shown in Figure 2A and results of a typical competition immunoassay experiment are shown graphically in Figure 2B.

RESULTS

Antigen competition of monoclonal antibodies. One mouse, immunized with a crude preparation of kappa carrageenan, produced antibodies with binding competed preferentially by either kappa, iota, lambda, or other carrageenan epitopes (Table 2). Each antibody had a characteristic immunoassay compe-

Table 2. *Antibody specificities by enzyme immunoassay competition. Relative amounts of kappa, iota, and lambda carrageenans needed for 50% reduction of antibody binding to kappa carrageenan, compared with a value of "1" for the best competitor. The second column shows the amount of antigen needed in micrograms per microtiter plate well for 50% competition by the best competitor for that antibody, either long kappa (κ), long iota (ι), or long lambda (λ).*

Antibody	μg/well	Long kappa	Long iota	Lambda	Short kappa	Short iota
6A11-2A4	1.1 (κ)	1	50	60	>9	>9
5H12	0.03 (κ)	1	3.6	600	1.6	8.8
7E4-3E9	0.6 (κ)	1	2.3	>33	3.7	>33
10A5	1.1 (κ)	1	1.6	69	1.8	8.6
7C2	0.03 (κ)	1	1.7	233	38	1296
7H6-3D12	0.3 (κ)	1	1.1	16	2	16.1
4D12-1A12	0.4 (ι)	2.9	1	1.9	>345	9
3G1	0.4 (λ)	11.9	2.3	1	153	4.9
3G1 + DL[a]		109	39	1		

[a] Diploid *Chondrus* lambda was used both to coat the plate (instead of long kappa) and as inhibitor.

tition pattern with samples representing different carrageenan types (Table 2). The patterns were consistent in multiple experiments. The amount of the best competitor needed to produce a 50% reduction was 0.03–1.1 μg per well in control binding for each antibody (Table 2). It should be remembered that the epitope recognized by a particular antibody may differ at least in part from the nominal sequence type used as competitor.

Antibody 6A11 binding was preferentially competed by a kappa-associated epitope (Table 2). It is unique in its strong preference for kappa over iota subunits. Because the primary difference between kappa and iota carrageenan is the sulfate group on carbon-2 of the 1–4-linked anhydrogalactose unit (Fig. 1), the 2-sulfate may interfere with binding. Although binding by this antibody was competed slightly more by short kappa than by short iota carrageenan fragments, 50% competition was not achieved even at high competitor concentrations. Acid hydrolysis during preparation of short carrageenan fragments may have modified an important aspect of the 6A11 epitope, although acid-hydrolyzed fragments were included in the immunizing preparation. Antibody 6A11 binding was not competed by beta carrageenan, the unsulfated backbone of kappa carrageenan, so the 4-sulfate on the 3-linked galactose may be an important aspect of the epitope.

Kappa carrageenan was also the best competitor for binding by antibodies 5H12, 7E4, 10A5, 7C2, and 7H6 (Table 2), and lambda carrageenan was a poor competitor. In contrast to antibody 6A11, these antibodies were competed almost as well by iota as by kappa carrageenan. Therefore, the epitopes for this group of antibodies are related to kappa and iota carrageenan, the gelling subunits. They were competed more by long kappa molecules than short kappa fragments, and more by short kappa than short iota fragments. Antibody 5H12 binding had the most differential competition, 600 times greater for kappa carrageenan compared with lambda carrageenan, and 7C2 was more than 200 times greater for kappa than lambda. Competition of antibody binding for 5H12, 10A5, and 7H6 by short carrageenan chains was nearly as great as that by the longer molecules. These three antibodies may have relatively short internal epitopes, because an increase in the proportion of chain ends had little effect, or helical conformation may not be involved in antibody recognition. Each of this group of five antibodies differs in the degree of competition of binding by lambda carrageenan, short fragments, and immunofluorescence localization patterns (see later).

Antibody 4D12 binding was unique in exhibiting greatest competition by iota carrageenan (Table 2). Its preference for iota over kappa was repeated and amplified with the short-chain kappa and iota samples. The sulfate on carbon-2 of anhydrogalactose may be important for binding by antibody 4D12, in contrast to antibody 6A11.

Antibody 3G1 was the only antibody for which the best carrageenan competitor was lambda carrageenan. The lambda > iota > kappa preference was repeated and amplified in the weaker binding of short-chain samples (Table 2). The order-of-magnitude preference for lambda over kappa carrageenan by 3G1 was increased almost another order of magnitude when both the plate-coating antigen and the competitor were diploid lambda carrageenan (Table 2). No protein was detected in the immunizing antigen, although the diploid lambda carrageenan preparation had a protein contaminant. Other possible minor components have not been excluded.

Oligosaccharide competition. Preliminary competitive enzyme immunoassay experiments were carried out with four purchased carrageenan oligosaccharides (see Materials and Methods section) used as competitors. These were (1–3)-linked dimers (neocarrabiose and neocarrabiose 4^1-sulfate) and (1–3)-linkage-centered tetramers (neocarratetraose $4^1,4^3$-disulfate and neocarratetraose 4^1-sulfate). No oligosaccharide competition was seen for binding of antibodies 6A11, 10A5, or 3G1 at 20 mM. This corresponds to 7000 times the amount needed for

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50% competition by long kappa for 6A11 and 10A5 binding, and 20,000 times the amount needed for 3G1 competition by diploid lambda carrageenan. Antibodies 7E4 and 7H6 were inhibited weakly only by the disulfated, kappa-type tetrasaccharide. A maximum of 10–15% competition was observed for antibody 7H6-3D12 binding at 2 mM of the disulfated tetrasaccharide. Binding of antibody 7H6-3B7 (a sister clone identical to antibody 7H6-3D12 binding by immunofluorescence pattern) was competed 50% by 1.5 mM of disulfated tetrasaccharide. The concentration of this tetrasaccharide needed for 50% competition was about 7000 times the amount needed for long kappa with this antibody. Similarly, antibody 7E4-3E4 binding was competed 50% by 0.64 mM of the disulfated tetrasaccharide, corresponding to 1500 times the value for long kappa. Kappa-type tetrasaccharides are clearly very poor competitors.

Developmental changes in Kappaphycus *tissues. Kappaphycus* tissues at six stages of development from 0.27 to 4.6 mm in diameter were stained with toluidine blue O for acidic carbohydrates and with Coomassie blue for proteins. Younger tissue was stained more intensely for both cell wall carbohydrate and intracellular protein (Figs. 3–10). Less-dense acidic wall material was seen in the medulla of the 4.6-mm section (Fig. 10), and little protein staining could be seen in the inner tissues of 4.6-mm sections. The higher concentration of acidic groups in the cell wall and increased protein content in the younger tissues is probably related to more rapid synthesis and growth. The cuticle stained most intensely with toluidine blue O (Figs. 3, 5, 7, 9), showing a high sulfate concentration at that location.

The epidermis is composed of one layer of slightly elongated cells up to a thallus diameter of 0.78 mm. The thickened outer cell wall of epidermal cells (i.e. cuticle) contains layers of wall material (Figs. 3, 7, 9). At the next stage examined (2.6 mm diam), the epidermis had increased to three layers of narrower, more elongated cells. No further change was seen at the 4.6-mm stage.

Medullary cell diameter increased 2 times between the 0.27- and 0.78-mm stages and increased an additional 5 times between the 0.78- and 2.6-mm stages (Table 3). This was a total increase of 10-fold (from 40 μm to 400 μm), with no further increase in diameter at the 4.6-mm stage. This corresponds to a 400–1000-μm diameter for medullary cells reported by Doty (1985). Wall thickness of medullary cells also increased in these embedded sections, although toluidine blue O staining intensity of walls decreased with development. More layers of medullary cells were added during development, from approximately two layers at the 0.27-mm stage to about 10 layers at the 4.6-mm stage.

The medullary core consisted of cells of smaller diameter embedded ...

TABLE 3. *Developmental changes in thickness of* Kappaphycus *tissues. Average radial thickness in micrometers of tissue type in the cylindrical thallus.*

Developmental stage (mm section diameter)	Epidermis	Medulla	Core
0.27	30	60	47
0.31	30	66	54
0.62	30	120	150
0.78	30	180	180
2.6	110	850	350
4.6	90	1700	500

material (Figs. 3, 4). Although core cells are considered to be secondary growth (Doty 1985, Doty and Norris 1985), they were found at the 0.27-mm stage, very near the growing tip, where they appeared to be dividing (Fig. 3). At this stage, a third of the thallus diameter consisted of core tissue. Core cells increased in size during development, although the medullary core was still increasing in diameter at the 4.6-mm stage (Table 3). Core tissue becomes less distinct as core cells enlarge and take on the appearance of medullary cells, so that the core is lacking in more mature stages of larger diameter (Doty 1985).

Thallus, tissue, and cell sizes were estimated by direct microscopic measurement and by measurement on negatives of equal magnification (Table 3). Increase in the diameter of the medulla is responsible for most of the increase in thallus size. A transition zone in cell files between the epidermis and medulla consists of spherical cells that gradually increase in size from epidermal cell diameter to medullary cell diameter (Figs. 3–10). This transition zone has several cell layers at the 2.6- and 4.6-mm-diameter stages, when the epidermis consists of about three layers of cells. At these stages, the number of medullary cell layers is increasing, and the diameter of medullary cells is 10 times larger than at the earliest stage examined. The source of the increase in number of medullary cells is clearly the dividing inner epidermal cells, which undergo a shape change and begin to enlarge. Therefore, this subsurface zone appears to be the primary region of cell wall production and modification.

Developmental changes in carrageenan antigen distribution. Immunofluorescence localization of carrageenan epitopes was examined on *Kappaphycus* sections during early tissue development at thallus diameters of 0.27 and 0.78 mm (Figs. 11–17), after the switch to a multi-layer epidermis at a diameter of 2.6 mm (Figs. 18–24), and in the more mature thallus at 4.6 mm diameter (Figs. 25–47). The peripheral meristematic region (Figs. 25–31), walls of 0.5-mm-diameter medullary cells (Figs. 32–39), and the medullary core (Figs. 40–47) were studied at the 4.6-mm-diameter stage.

Immunofluorescence localization on sections of mature *Kappaphycus* thallus confirmed ...

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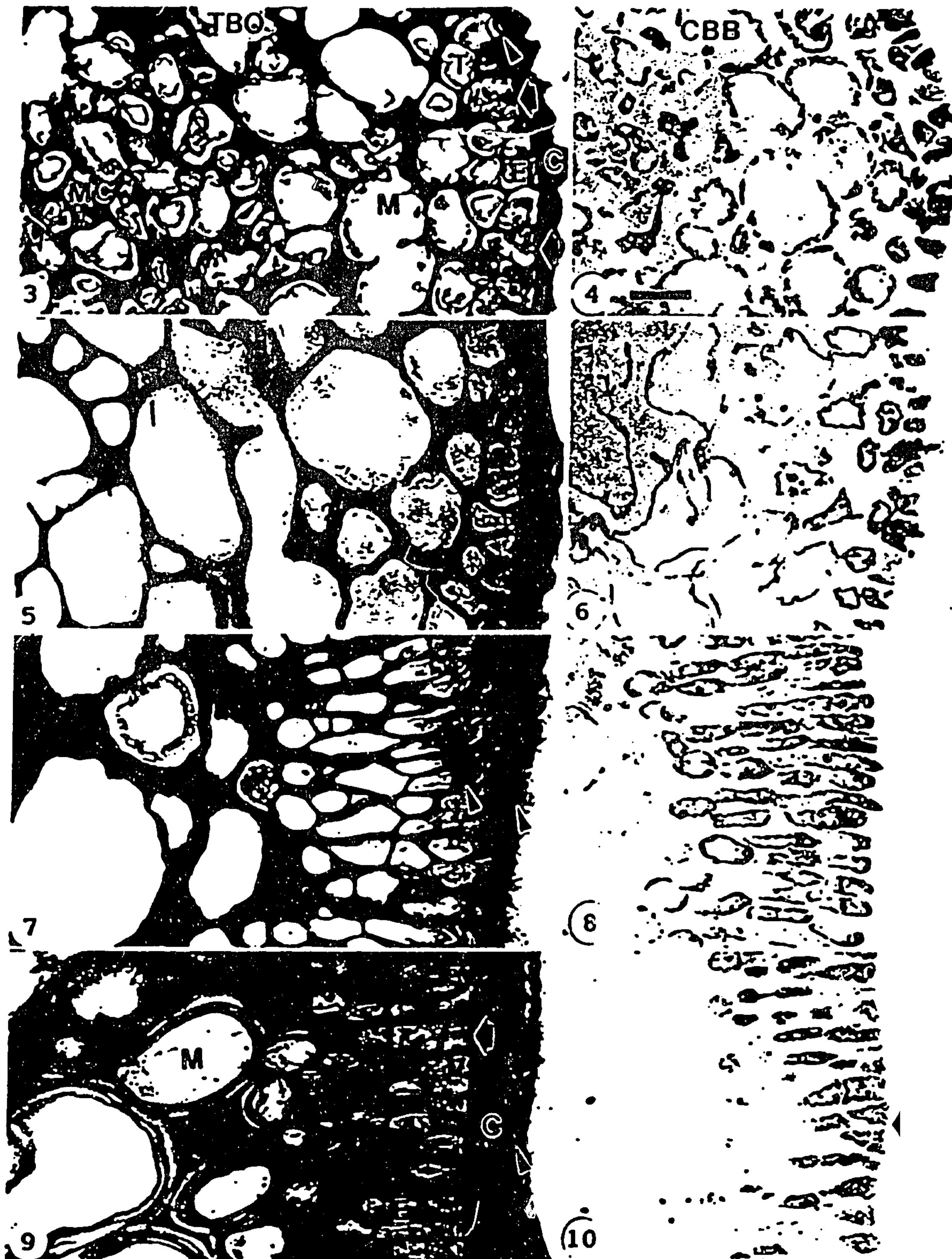

Figs. 3–10. Developmental changes in *Kappaphycus* tissues during vegetative growth. Toluidine blue (TBO) staining of carrageenan (left column) and Coomassie brilliant blue (CBB) staining of protein (right column). Thallus diameter: Figs. 3, 4. 0.27 mm. Fig. 5. 0.62 mm. Fig. 6. 0.78 mm. Figs. 7, 8. 2.6 mm. Figs. 9, 10. 4.6 mm. Arrowheads in Figures 3, 7, and 9 indicate carrageenan layers deposited in cuticle. Solid arrows in Figures 3, 9, and 10 show division of epidermal cells producing branched cell files. Hollow arrow in Figure 3 shows a dividing core cell. C, cuticle; E, epidermal cell; T, transition zone; M, enlarging medullary cell; MC, medullary core. Scale bar = 43 μm in Figure 4 is magnification for Figures 3–10.

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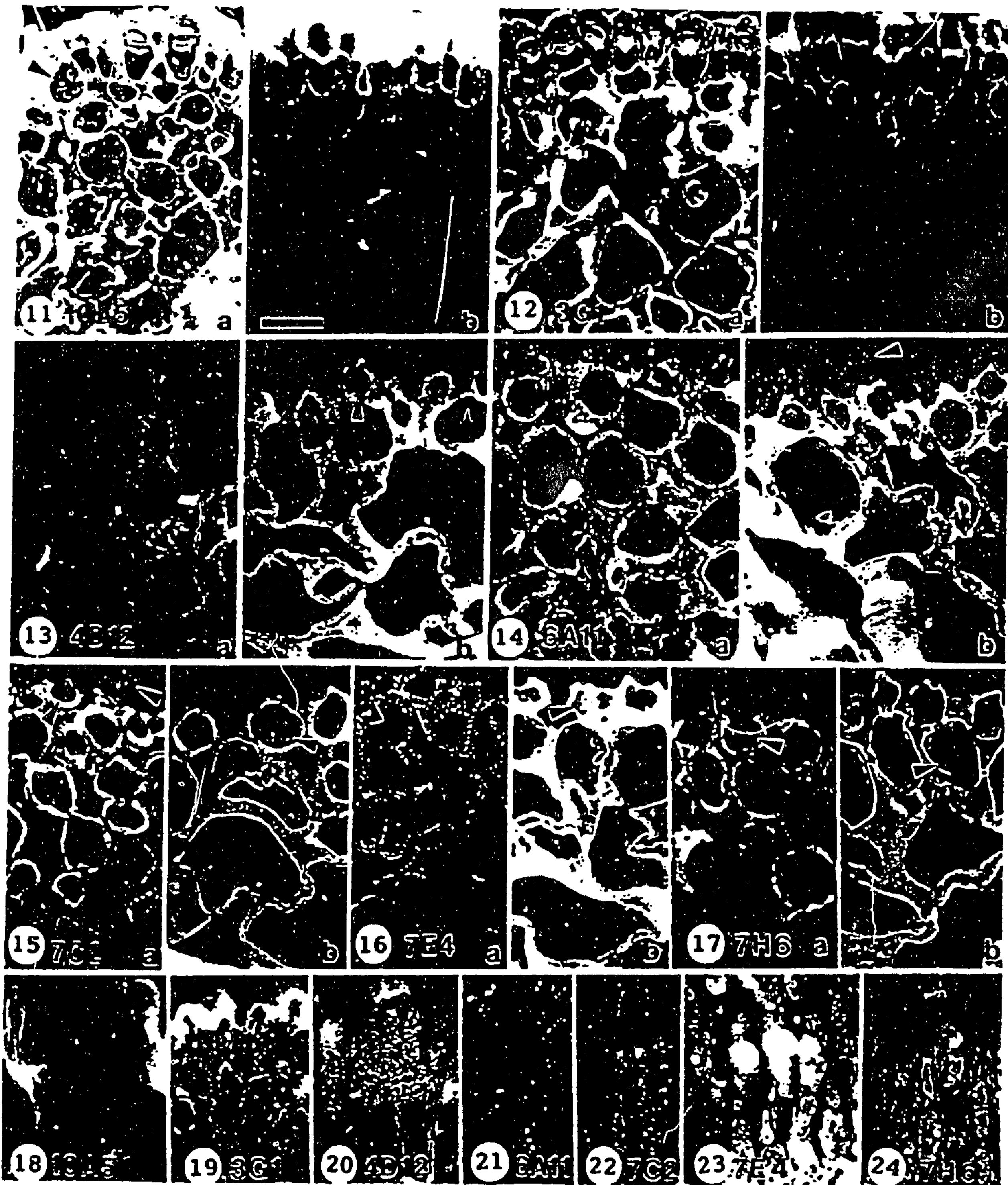

FIGS. 11–24. Immunofluorescence labeling by the antibodies as indicated at the thallus surface at three developmental stages. FIGS. 11a–17a. 0.27-mm-diameter sections (0.31 mm in Figs. 14a and 17a). FIGS. 11b–17b. 0.78-mm-diameter sections. Arrowheads indicate intracellular labeling. Asterisks in Figures 11 and 12 show carrageenan in cuticle layers. FIGS. 18–24. Epidermal cells in 2.6-mm-diameter sections. Asterisk in Figure 20 shows initiation of carrageenan accumulation in the outermost epidermal cell (see inner arrowhead in Fig. 7). Scale bar = 43 μm in Figure 11b is magnification for Figures 11–24.

25–47). Each antibody labeled extracellular carrageenan with a unique and characteristic labeling pattern. The possibility that variations in antibody concentration in hybridoma supernatants caused labeling differences was examined. First, antibody supernatants were diluted into hybridoma medium prior to immunofluorescence. Second, supernatants from subclones of the same antibody, which prob-

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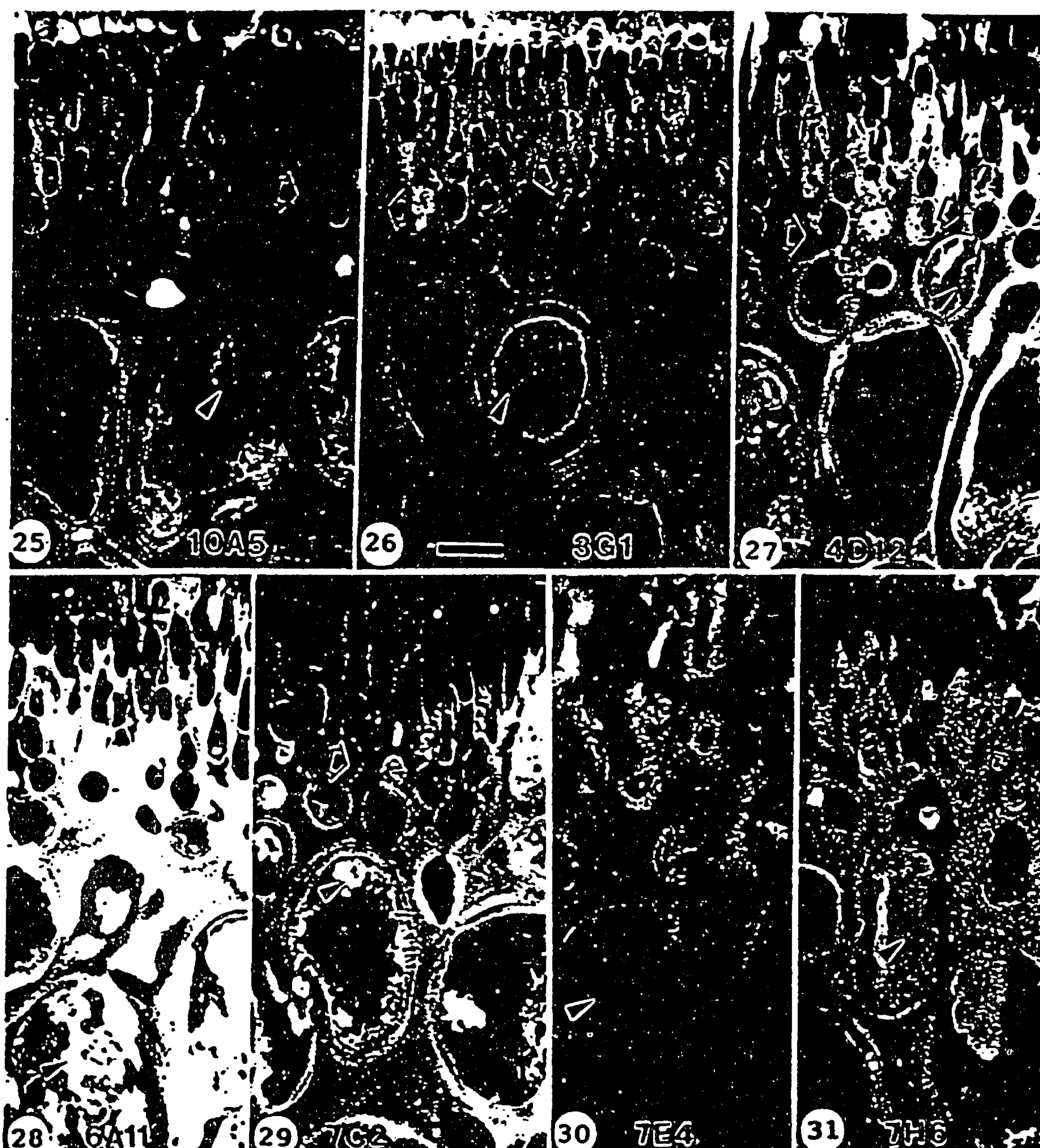

Figs. 25–31. Immunofluorescence by the indicated antibodies at the thallus surface of sections 4.6 mm in diameter. Arrows show change in wall pattern from epidermal type (thin) to medullary type (layered and thicker) in the transition zone. Arrowheads show intracellular accumulation of carrageenan in enlarging medullary cells. Asterisks in Figures 25 and 26 show carrageenan in cuticle layers. Scale bar = 43 μm in Figure 26 is magnification for Figures 25–31.

ably contained different antibody concentrations, were used for immunofluorescence. The characteristic labeling pattern of each antibody was maintained in both types of experiments, demonstrating that differences in antibody concentration did not contribute to different localization patterns. The antibodies were organized into two groups according to their immunofluorescence labeling patterns.

The first group of antibodies (3G1 and 10A5) is distinguished by a combination of cuticle labeling and a decrease in labeling during early development (Figs. 11, 12). These antibodies label the cuticle intensely and in a layered pattern. They labeled medullary cell wall and extracellular matrix regions primarily in undulating lines resembling topographical maps (Figs. 33, 34). Antibody 10A5 labeling decreased more rapidly during development, and this antibody labeled intracellular material more prominently, especially at the earliest stage of development (Figs. 11, 12). Antibody 3G1 labeled small cells between the large medullary cells (possibly thylle cells: see Weber-van Bosse 1928; Figs. 33, 34) most

walls of the adjacent transition cells were labeled (Figs. 13–17, 27–31). This labeling pattern could be explained by masking of the epitopes in the epidermal wall but not the transition cell wall. Since epidermal and transition cell walls are both highly sulfated, differential masking is unlikely. It is more likely that carrageenan bearing these epitopes is packaged in intracellular vesicles but not secreted until the transition cells become differentiated from epidermal precursor cells. In this case, the signal to secrete carrageenan would be closely related to the cell type switch. Polarized wall labeling of carrageenan types is seen in epidermal cells at the 4.6-mm stage (Figs. 27–31), where lateral epidermal walls but not the cuticle are labeled by these antibodies.

High concentrations of some of the antibodies caused localized wall swelling, especially in younger tissues (Figs. 11–24). The swollen areas decreased in size and number when antibody was diluted in hybridoma supernatant and when sections were postfixed prior to antibody incubation. Wall swelling provides evidence that these antibodies bind to gelled carrageenan and that antibody binding disrupts wall structure.

In summary, carrageenan production in *Kappaphycus* was observed primarily in the peripheral meristematic region consisting of the epidermis and transition zone. Intracellular carrageenan antigens appeared punctate in epidermal cells, then in larger inclusions in transition cells, and finally filled the apparent vacuole of partially enlarged medullary cells, although no intracellular labeling was seen in fully enlarged medullary cells of these cultured thalli. This progression appears to represent temporary intracellular accumulation of carrageenan to provide wall material needed during the 10-fold enlargement of medullary cells. In younger tissue, gelling carrageenan was produced in epidermal cells but apparently not incorporated into the wall until differentiation of the first transition cell. After a transition cell was separated from the epidermal cell by cell division, incorporation of gelling subunits into the wall was observed (Figs. 13–17). The cell type switch also involved a change in the type of wall produced from the thin epidermal wall to the layered medullary wall (Figs. 25–31). In addition to producing carrageenan for thallus growth, the epidermal cells produced highly sulfated carrageenan in the cuticle by polarized secretion. Epidermal cells at the 2.6-mm stage were especially active in carrageenan production (Figs. 18–24). At this stage, increase in cuticle thickness by filling the space previously occupied by the outermost epidermal cell with successive layers of carrageenan was seen (Figs. 7, 20). This resulted in the thick, layered cuticle seen at a later stage (Fig. 9). The production of gelling carrageenan precursor was greatest in the youngest tissue. The concentration of kappa carrageenan was highest throughout development, as expected for *Kappaphycus*. The anti-carrageenan antibodies did not label sections of the agar-producing red algae, *Gelidium* and *Gracilaria*.

DISCUSSION

Antibody specificity for carrageenan subunits. Comparison of competition and localization patterns showed that each of the eight antibodies recognized a different epitope. Antibodies 3G1 (lambda-related) and 10A5 (precursor-related) were the only ones to label the cuticle. The epitope for 4D12 was iota-related. The epitopes for six antibodies were most closely associated with kappa and were distinguished in other ways. Antibody 6A11 binding was competed by kappa much more than by iota, was poorly inhibited by short fragments, and produced the strongest extracellular labeling. Binding by antibodies 5H12 and 7C2 was competed well by kappa and iota compared to lambda, although 5H12 binding (but not 7C2) was competed well by short fragments. Three antibodies, 10A5, 7E4, and 7H6, showed similar competition patterns. These antibodies differed considerably in immunofluorescence labeling patterns: the 10A5 labeling pattern differed radically from 7E4 and 7H6, whereas the latter two antibodies were distinguished by the characteristic granular appearance of labeling by 7H6. The 10A5 epitope may include a precursor to anhydrogalactose in gelling carrageenans, because intracellular labeling by 10A5 was correlated temporally and spatially with carrageenan production.

The nonhomogeneous nature of carrageenan minimized the apparent degree of specificity in enzyme immunoassay data (Table 2). For example, with kappa and iota samples each containing 10% of the other type of carrageenan, differential antibody binding of ninefold greater for one sample would be expected for a totally specific antibody. The kappa sample used in the present study contained some iota sequences, which were detected by infrared spectroscopy and contributed to a sulfate content 14% higher than expected (Table 1). Even with the heterogeneous samples employed, antibody 6A11 exhibited a 50-fold preference for kappa over iota. In addition, antibody 3G1 exhibited a 100-fold preference for lambda over kappa when the more homogeneous diploid lambda was used for both solid phase and competitor in the enzyme immunoassay. This carrageenan from diploid *Chondrus* plants nevertheless contained about 2% anhydrogalactose, a component of idealized kappa and iota but not lambda structure. Detailed antibody specificity studies using well-characterized carrageenan fragments prepared with specific carrageenases are needed for further studies of carrageenan epitopes.

All antibodies showed greater competition by antigens that had a lower proportion of chain ends to internal sugar units (long vs. short carrageenans; Table 2). The differences in competition were amplified with short fragments as inhibitors, but (1–3)-centered, kappa-type tetrasaccharides were poor

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competitors. The antibodies may all recognize internal sites on carrageenan chains. Alternatively, helical conformation of carrageenan may be needed for antibody recognition but is lacking in tetrasaccharides and possibly a significant fraction of the molecules in the short carrageenan preparations. The (1-4)-centered tetrasaccharide was not available for testing but may have a more appropriate linkage sequence for the epitopes.

All of the monoclonal antibodies labeled the carrageenan in cell walls of a kappa-producing red alga. Wall swelling due to antibody interaction was evidence that antibody labeling disrupted wall organization. This contrasts to the competition of binding by anti-alginate monoclonal antibodies by steric hindrance, due to strong gelling interactions in brown algal cell walls (Vreeland et al. 1984). The conformation of carrageenan polymers and their interactions are highly dependent on ionic strength and salt composition (Rochas and Rinaudo 1980, Morris and Belton 1982, Smidsrød and Grasdalen 1984). Labeling was carried out in hybridoma medium. Although carrageenan is a highly sulfated polymer that can bind to proteins by electrostatic interactions (Glicksman 1983), no nonspecific binding was seen at the incubation conditions used this study. For example, although sulfate is concentrated at the plant surface as shown by toluidine blue staining as well as by two antibodies, the other eight antibodies did not label this region.

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The use of antibodies with a variety of specificities is needed for a full characterization of carrageenan composition. Antibody 6A11 with kappa-related specificity together with antibody 4D12 with iota-related specificity might be useful for quantitative kappa/iota content analysis. Several antibodies bind to both kappa and iota gelling subunits and could provide a measure of total gelling subunit content. Antibody 3G1 detects highly sulfated nongelling carrageenan, and antibody 10A5 labeling reflects the amount of precursor. These antibodies do not label agar-producing algae. Therefore, the anti-carrageenan antibodies distinguish which type of wall galactan is produced by a particular red alga.

Carrageenan synthesis and modification. The results presented here support the intracellular synthesis of carrageenan. Discrete labeled structures were seen within most cell types, especially in epidermal and transition cells. Intracellular iota subunits were identified by antibodies 4D12 and 7E4 in ultrastructural immunogold localization on growing apices of *Agardhiella subulata* (Gretz et al. 1990). Carrageenan was labeled in Golgi vesicles as well as in the cell wall at the apical meristem. The localization of carrageenan subunits in punctate intracellular inclusions in the present study may also represent Golgi vesicles.

The nature of the sulfation and anhydrogalactose production processes in red algal polysaccharides is controversial. Enzymes from carrageenan-producing plants, evidently transferases that cause cyclization by removing the 6-sulfate from D-galactose to form 3,6-anhydrogalactose, have been found in cell-free extracts of *Gigartina stellata* (Lawson and Rees 1970) and *Chondrus crispus* (Wong and Craigie 1978). *Porphyra umbilicalis*, which contains an agar-type polymer, has a similar enzyme that removes the 6-sulfate from L-galactose with concomitant formation of the 3,6-anhydrogalactose (Peat and Rees 1961, Rees 1961a, b). Millard and Evans (1982) showed the presence of sulfate in the Golgi of the unicellular red alga, *Rhodella maculata*, which produces a sulfated capsular polysaccharide. They found no evidence of extracellular sulfatase or sulfotransferase in *Rhodella*.

In contrast, in an autoradiographic study with *Eucheuma nudum*, LaClaire and Dawes (1976) found no evidence for cytoplasmic incorporation of the labeled sulfate. Sulfate was first seen in the part of the wall adjacent to the cell and was ultimately deposited in the outer epidermal wall and middle lamella. This extracellular distribution in mature tissue is similar to the labeling patterns of antibodies 3G1 and 10A5 in our study, where intracellular labeling was also seen with these antibodies in epidermal cells. LaClaire and Dawes (1976) suggested that the rapid uptake of the label indicated sulfate addition to preexisting polysaccharide in the wall, although loss of soluble intracellular carrageenan was also a possibility. Extracellular sulfation would differ from results for animal cells (Young 1973) and for brown algal cells (Evans et al. 1973, Callow and Evans 1976, Callow et al. 1978, Brawley and Quatrano 1979), where sulfate incorporation in Golgi vesicles was interpreted to represent sulfation of fucan polysaccharides. However, sulfate incorporation into carbohydrates in the Golgi apparatus has not been firmly established for brown algae because sulfated phenolics are produced by brown algal cells (Ragan and Jensen 1979) in addition to sulfated fucans and could also be sulfated in the Golgi apparatus. The intracellular labeling by antibody 6A11, which was not inhibited by unsulfated kappa carrageenan, supports the intracellular sulfation of kappa carrageenan.

As found here for *Kappaphycus*, a high concentration of carrageenan precursors is expected in younger tissue. Tian et al. (1988) found that the anhydrogalactose content of *Eucheuma* species increases with tissue age, and Craigie and Wen (1984) showed that there are more precursor units to the anhydrogalactose of agar in younger portions of *Gracilaria* thalli. Vreeland et al. (1992) found accumulation of carrageenan epitopes within the lumen of large medullary cells on immunolabeled tissue prints of *Kappaphycus* sections.

Comparison of antibody and hybridization probe labeling patterns. The distribution pattern for kappa carrageenan in *Kappaphycus*, which was produced by antibody 6A11, was very similar to the pattern pro-

duced by another kind of molecular marker, the kappa gelling probe (Zablackis et al. 1991). Antibody 6A11 and the kappa probe both labeled walls more strongly than matrix except in the transition zone, where wall and matrix were labeled equally. Both also labeled strongly in young as well as older tissue and did not label cuticle or epidermal walls. This supports the specificity of both types of molecular markers for kappa subunits. However, the labeling pattern of the antibody 4D12 did not consistently correspond to the iota probe pattern. On some sections of larger diameter thalli, the iota antibody pattern was similar to the iota probe in its weak labeling of medullary matrix but not walls. On other sections with more concentrated antibody, the walls were also labeled, and the matrix appeared swollen. This difference may be explained by concentrated antibody contributing to iota solubility from the matrix and the greater antibody sensitivity enabling wall labeling. The other main difference between the iota antibody and probe was in cuticle labeling. The probe labeled the cuticle, but the antibody only labeled incipient layers of the cuticle at one developmental stage (Fig. 20). Another antibody (10A5) with possible specificity for iota precursor did label the cuticle. This difference serves to emphasize that epitopes recognized by the antibodies may not correspond fully to the strictly regular structures of idealized carrageenan gelling subunits.

In summary, localization of carrageenan epitopes in peripheral meristem and enlarging medullary cells showed the intracellular synthesis of carrageenan at the vegetative apex of *Kappaphycus* branches. The extracellular distributions of carrageenan epitopes differed among cell types and revealed a cell type switch early in the development of medullary cells from meristematic cells.

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