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Partial Purification and Characterization of a Glycoprotein Cell Fusion Hormone from *Griffithsia pacifica*, a Red Alga[1]

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ABSTRACT

In filaments of the red alga *Griffithsia*, dead intercalary cells are replaced by the process of cell repair by cell fusion. This process is coordinated by a morphogenetic cell fusion hormone, rhodomorphin, which accelerates cell division and induces the production of a specialized repair cell. We have isolated rhodomorphin from *Griffithsia pacifica* Kylin and have purified it by concanavalin A affinity chromatography, hydrophobic interaction chromatography, and gel filtration chromatography. This molecule binds specifically to concanavalin A, is proteinase sensitive, and is inactivated by short treatments at temperatures of 50°C or above. It therefore appears that rhodomorphin from *G. pacifica* is a glycoprotein; its molecular weight, as estimated by gel filtration, is approximately 14,000.

Chemical communication to ensure simultaneous sexual development of compatible partners and to facilitate sexual cell fusion occurs in many organisms. Among the eukaryotic microbes, a number of very different compounds have been shown to function as sexual inducers (11). These include steroids in the oomycete *Achlya* (1, 8); carotenoids in certain Mucoralean fungi (3); oligopeptides in some Ascomycete and Basidiomycete yeasts (2, 12, 16); and glycoproteins in the green alga *Volvox carteri* (5, 6, 14). We are studying a developmental process which can be induced in filamentous red algae in the genus *Griffithsia* which leads to vegetative cell fusion. This process, cell repair, is coordinated by a morphogenetic substance produced by one of the cells which participates in the fusion process (20)

In filaments of the marine red alga, *Griffithsia*, cell repair by cell fusion can be induced by killing an intercalary cell in a filament. About 5 h after cell death, the cell above the dead cell divides to produce a rhizoid cell. Stimulated by a morphogenetic substance from this rhizoid, the cell below the dead cell produces a specialized repair shoot cell. The rhizoid cell and the repair shoot cell grow together and fuse to form a single shoot cell, thus restoring the integrity of the filament (19, 20). We have shown that, in at least three species of *Griffithsia*, growing rhizoids produce a species-specific morphogenetic substance which induces freshly decapitated shoot filaments to make repair shoot cells. This substance is required to maintain the unusual morphology and growth pattern of repair shoot cells; it may also be involved in the attraction of the repair shoot to the rhizoid for fusion (17, 19, 20). While cell repair usually occurs between cells of the same plant, cell fusion can be induced between a rhizoid from one plant and a repair shoot from another plant (17, 18).

We have called this morphogenetic cell fusion hormone, 'rhodomorphin' (19, 20). Recently, we have described a method for obtaining quantities of crude rhodomorphin from *G. pacifica* (20). In this paper, we will describe the partial purification and chemical characterization of rhodomorphin from *G. pacifica.*

MATERIALS AND METHODS

Chemicals. Biogel A 1.5m (100–200 mesh), Bio-Gel P-2 (100–200 or 200–400 mesh) and Bio-Gel P-6 (100–200 mesh) were obtained from Bio-Rad Laboratories. Acrylamide, bisacrylamide, glycine, *N,N,N',N'*-tetramethylenediamine, and ammonium persulfate for PAGE[2] were also obtained from Bio-Rad Laboratories. Lectins immobilized on agarose were obtained from Sigma Chemical Co.; these lectins included Con A, *Ulex europeus* lectin, wheat germ lectin (on Sepharose 6 MB), and *Arachis hypogaea* lectin. Also obtained from Sigma Chemical Co. were polyethylene glycol 6000 (PEG 6000), Tris base, BSA (Sigma fraction V), α-methyl-D-mannoside (Sigma grade I), kanamycin sulfate, PMSF, SBTI, L-fucose, 1-*O*-methyl-β-D-galactopyranoside, *N*-acetyl glucosamine, octyl agarose (amino-linked), and guanidine-HCl. Guanidine-HCl (Sigma grade I), was used without further purification; practical grade was filtered through a Millipore HA15 glass prefilter prior to use. All Millipore products were obtained from Millipore Corporation. Ampicillin (Polycillin N) was obtained from Bristol Laboratories, Syracuse, NY; gentamicin sulfate (Garamycin, injectable) from Schering Corp., Kenilworth, NJ, and streptomycin sulfate from Pfizer Laboratories. Proteinase K was obtained from Merck and Co., and trypsin from Worthington.

Organism. The alga used in this study is the giant-celled, filamentous, marine, red alga *Griffithsia pacifica* (Rhodophyta, Ceramiales). This organism was grown as described previously (20) in modified f/2-enriched sea water (f/2) at 20°C on a 16-h light:8-h dark cycle under 5.5 to 7.0 nmol· $cm^{-2}\cdot s^{-1}$ cool-white fluorescent light.

Preparation of Crude Hormone Solutions. Crude solutions of rhodomorphin were obtained as described previously (20). Briefly, for each hormone preparation, algal filaments were chopped into segments of 1 to 5 cells. These segments were shaken for 1 h in f/2-enriched seawater with added antibiotics (100 μg/ml) ampicillin and 100 μg/m; streptomycin). This and all subsequent incubations were carried out at 20°C and continuous 3 nmol· $cm^{-2}\cdot s^{-1}$ cool-white fluorescent illumination. After 1 h, the filaments were transferred to f/2 containing antibiotics plus 100 μg/ml BSA. They then were shaken for 24 h during which time each segment regenerated a rhizoid at its base and produced rhodomorphin. Filaments were then removed and the hormone-containing medium was filtered through a Millipore AP15 glass prefilter and a Millipore GSTF (0.22-μm pore) filter. At this point, the

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[2] Abbreviations: Con A, concanavalin A; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; Con A-agarose, Con A immobilized on agarose beads.

crude rhodomorphin solution could be stored at −16°C or used directly in further purification steps.

Assay for Biological Activity. The method used to determine the specific activity of putative hormone solutions has been described in detail elsewhere (20). Briefly, the ability of rhodomorphin to induce decapitated shoot filaments to produce repair shoots was used as the biological assay for rhodomorphin activity. To determine the specific activity of a given solution, a 2-fold dilution series was prepared in f/2 containing 100 μg/ml BSA and 40 μg/ml gentamicin or kanamycin. To each dilution were added assay filaments with at least eight decapitated apices; these were shaken for 24 h under the conditions described above for hormone isolation. After 24 h, the number of regenerating apices which had made repair shoots was recorded. The most dilute solution in which 50% of the regenerating apices formed repair shoots was defined to have one unit of activity per ml. Inasmuch as dilutions were done in a 2-fold series, estimates of specific activity have an uncertainty of about 25%.

Dialysis and Buffer Exchange. In early experiments, rhodomorphin activity was exchanged into different buffers by dialysis. However, as the purification of rhodomorphin progressed, it became apparent that absorption of hormone to cellulose, dextran beads, and other materials was causing loss of activity, particularly at low ionic strength. Therefore, all later buffer exchanges were done using short columns of Bio-Gel P-2 or P-6. All buffer exchanges were done by this latter method unless otherwise mentioned.

Lectin Affinity Chromatography. To determine whether rhodomorphin has lectin-binding sites, lectin affinity chromatography was carried out on small (0.7-cm diameter) columns, containing 0.5 to 1.0 ml of one of several lectins immobilized on agarose beads. The concentration of the immobilized lectins used was as follows: *U. europeus* (gorse) lectin, 0.58 mg/ml; *A. hypogaea* (peanut) lectin, 1.22 mg/ml; wheat germ lectin, 5.0 mg/ml; and Con A, 9.6 mg/ml. Onto each column were loaded 10 ml of crude hormone solution; the column was then washed with 2 ml of high-salt solution (2.0 M NaCl, 0.02 M Tris, pH 8.0). The load run-through and the high-salt wash were combined and designated as the 'unbound fraction.' Hapten-specific elution was carried out using 3 ml of a 0.5 M solution of the appropriate hapten in 2.0 M NaCl, 20 mM Tris, pH 8.0. To avoid any interference from the free haptens, these eluates, designated as the 'bound fractions,' were buffer exchanged on a Bio-Gel P-6 column into 0.5 M NaCl, 20 mM Tris, pH 8.0, prior to their assay for activity. All manipulations, prior to buffer exchange, were carried out at 4°C.

Preparative chromatography of crude rhodomorphin solutions was carried out on short columns (1–3 cm high and 1 cm diameter) of Con A-agarose. An entire crude hormone preparation (300–500 ml) was passed over the Con A-agarose column at a flow rate of about 0.5 ml/min at 4°C. The column was then washed with 7 ml of 2.0 M NaCl, 20 mM Tris, pH 8.0, to remove nonspecifically adsorbed substances. Specifically-bound substances were then eluted with 7 ml of a solution of 0.5 M α-methyl-D-mannoside, 2.0 M NaCl, 20 mM Tris, pH 8.0.

Protein Assay. Protein concentration was measured by the Amido Black 10B dye binding method (13).

Hydrophobic Interaction Chromatography. Bound fractions from the Con A column (in 0.5 M α-methyl-D-mannoside, 2.0 M NaCl, 20 mM Tris, pH 8.0) were further purified by chromatography on a 0.9 × 15 cm column of octyl agarose. The NaCl concentration of the rhodomorphin solution was raised to 4.0 M by the addition of solid NaCl. This solution was loaded, at a flow rate of 0.2 to 0.4 ml/min, onto an octyl agarose column which had been equilibrated in 4.0 M NaCl, 20 mM Tris, pH 8.0; this was followed by 5 ml of equilibration buffer. Bound fractions then were eluted by a 150 ml linear NaCl gradient (4–0.4 M NaCl in 20 mM Tris, pH 8.0) which was applied at a flow rate of 0.1 to 0.2 ml/min. Five-ml fractions were collected and directly tested for biological activity. All operations were performed at 20°C.

Gel Filtration Chromatography. Samples to be chromatographed were reduced in volume by one of two methods. In early experiments, samples were dialyzed against PEG 6000 or sucrose to remove most of the buffer volume; this step was followed by extensive dialysis against 0.5 M NaCl, 20 mM Tris, pH 8.0. Crystalline guanidine-HCl (Sigma grade I) was added to bring the guanidine-HCl concentration of the samples to 6.0 M. In later experiments, the volume of solutions was reduced by lyophilization. Before lyophilization, samples were buffer exchanged into 0.5 M NH_4HCO_3. Samples were lyophilized to dryness and the dry powders dissolved in 1 to 2 ml of 6.0 M guanidine-HCl, buffered with 20 mM Tris at pH 8.0 or 20 mM Na-acetate at pH 4.5.

The sample to be chromatographed was then loaded onto a 1.5 × 85 cm column of Bio-Gel A 1.5m, equilibrated in 6.0 M guanidine-HCl, buffered as above, and containing .05% NaN_3. Fractions of 4 ml each were collected. An aliquot (0.5–2.0 ml) of each fraction was removed to be assayed for biological activity. To each aliquot was added 100 μg BSA (Sigma fraction V) in 0.1 ml of f/2. Guandine-HCl was removed from each aliquot by buffer exchange into f/2. The specific activity of each aliquot was then assayed as described above.

PAGE. Samples for electrophoresis were exchanged into 0.5 M NH_4HCO_3; they were then lyophilized to dryness. SDS-PAGE on 15% gels was performed according to the method of Laemmli (7).

Gels were stained by three different methods. Two different silver stains for protein were used; these were done following the methods of Oakley *et al.* (10) and of Morrisey (9). Both techniques allowed the detection of bands containing as little as 1 ng protein. Gels were also stained for carbohydrate using the silver-periodic acid method of Dubray and Bezard (4).

RESULTS

Purification of Rhodomorphin. Crude rhodomorphin solutions were obtained by filtering medium containing a dense suspension of filaments with actively growing rhizoids. Compared to whole tissue homogenates, such solutions were enriched in morphogenetic activity and had reduced amounts of contaminating cytoplasmic proteins such as the photosynthetic phycobiliproteins.

Lectin Affinity Chromatography. Binding of rhodomorphin activity by various plant lectins was investigated using affinity chromatography on short columns of lectins immobilized on agarose beads. The lectins used and the sugars which they specifically bind are listed in Table I. The results of a typical binding experiment are also given in Table I. Of the four lectins tested, only Con A bound an appreciable amount of rhodomorphin. The lower amount of activity recovered from the Con A-agarose column is probably due to an incomplete elution of rhodomorphin by the hapten solution.

Since rhodomorphin is bound specifically by Con A, affinity chromatography on Con A-agarose was used as the first step in the purification of rhodomorphin. Table II shows the results of a typical preparative run on Con A-agarose. Activity from 500 ml of crude hormone solution was retained on a 2 ml Con A-agarose column. It was not significantly eluted by a 2.0 M NaCl wash. Most of the loaded rhodomorphin activity was, however, eluted by a wash containing the sugar hapten α-methyl-D-mannoside. The data in Table II show that Con A affinity chromatography of crude hormone solutions results in the removal of a high percentage of the contaminating proteins and in a 50-fold decrease in the volume of the rhodomorphin-containing solution. Typically, 50 to 100% of the rhodomorphin activity loaded on Con A-agarose columns was eluted in the hapten-containing wash. Hormone solutions purified and concentrated by Con A affinity chromatography are henceforth referred to as 'Con A-purified rhodomorphin.'

Table I. *Affinity Chromatography on Agarose-Immobilized Lectins*

Crude rhodomorphin-containing solution (1,200 total units activity) was passed over short columns of agarose-immobilized lectins; the columns were washed with a high-salt solution. The column run-through and the high-salt wash were combined and were designated as unbound. Specifically bound substances were eluted with a solution of the appropriate hapten; these fractions were designated as bound.

Lectin and Specificity	Specific Eluant	Unbound Fraction Activity Eluted	Bound Fraction Activity Eluted
		total units	
Con A (α-D-glucose, α-D-mannose)	α-Methyl-D-mannoside	100	300
A. hypogea (β-D-galactose)	1-*O*-Methyl-β-D-galactoside	1,200	0
U. europeus (L-fucose)	L-Fucose	1,200	0
Wheat germ (*N*-acetyl-D-glucosamine)	*N*-Acetyl-D-glucosamine	1,200	0

Table II. *Preparative Chromatography on Con A*

Crude hormone solution was passed over a column of Con A-agarose. Five ml of 0.1 M NaCl, 20 mM Tris (pH 8.0) was then loaded on the column. Seven ml of 2.0 M NaCl, 0.2 M Tris (pH 8.0) were passed through the column and the eluate collected (salt eluate). Finally, a wash of 0.5 M α-methyl-D-mannoside, 2.0 M NaCl, 20 mM Tris (pH 8.0) was passed over the column and the eluate collected (α-methyl-D-mannoside eluate).

Sample	Volume	Specific Activity	Total Activity	Recovered	Total Protein
	ml	*units/ml*	*units*	*%*	*μg*
Load	500	40	20,000	100	41,000
Run-through	505	<5	<2,500	<12	40,000
Salt eluate	7	40	280	1.4	4.9
α-Methyl-D-mannoside	7	1,500	10,500	52.5	33.6

Rhodomorphin activity was eluted from Con A by α-methyl-D-mannoside only when the elution buffer also had a high ionic strength. In fact, best recovery of rhodomorphin activity in all experiments was obtained if all manipulations were carried out at high ionic strength (>0.2 M). In low ionic strength buffers, rhodomorphin binds to a number of substances, including cellulose and dextran polymers.

Gel Filtration Chromatography. A second purification method employed was gel filtration which gave an estimation of mol wt in addition to providing substantial purification. Several Con A-purified rhodomorphin preparations were concentrated and chromatographed on Bio-Gel A 1.5m under denaturing conditions; 6.0 M guanidine-HCl was used as the chaotropic agent. Biologically active rhodomorphin was readily recovered from 6 M guanidine-HCl solutions by either dialysis or buffer exchange into f/2.

Figure 1 shows a typical elution profile for rhodomorphin activity. Rhodomorphin activity eluted as a single peak in an elution volume corresponding to a mol wt of 12,500 to 14,500 D. Typically, 60 to 100% of the loaded activity was recovered after this purification step. It was necessary to include the chaotrope, 6 M guanidine-HCl, in the chromatographic buffer to obtain this sharply defined peak of eluted activity. If gel filtration was done in its absence, under nondenaturing conditions, rhodomorphin activity eluted in multiple peaks (data not shown). This was presumably due to aggregation of rhodomorphin molecules and/or to their binding to other macromolecules present in these preparations.

The degree of purification obtained by gel filtration chromatography was monitored by SDS-PAGE (Fig. 2). The active fractions from gel filtration have three major bands which stain for protein: bands I, II, and III; there are also a few minor bands. Bands I and II are always present in active fractions following the above purification protocol. These bands run at a position corresponding to mol wt of 14,000 and 15,000 D. Each of the bands in the lane containing protein standards contains 20 ng protein.

Hydrophobic Interaction Chromatography. Hydrophobic interaction chromatography on octyl agarose was used as another step in the purification of rhodomorphin. Con A-purified rhodomor-

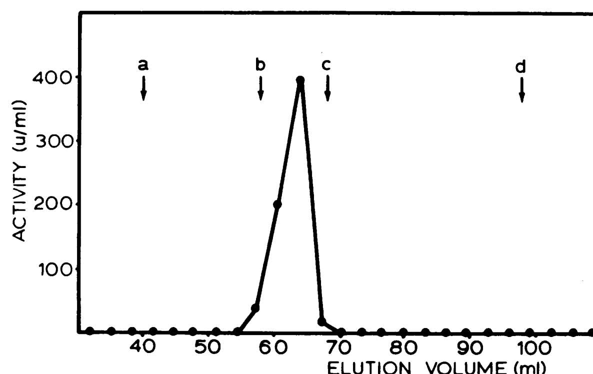

FIG. 1. Gel filtration chromatography of Con-A-purified rhodomorphin. Rhodomorphin-containing solution (3,000 units activity), concentrated and purified by Con A-agarose affinity chromatography, was chromatographed on Bio-Gel A 1.5m beads in the presence of 6.0 M guanidine-HCl. Elution volumes of marker proteins, used to calibrate the column, are indicated by arrows: (a), BSA (68,000); (b) myoglobin (17,500); (c), Cyt *c* (12,400); and (d), insulin (5,800).

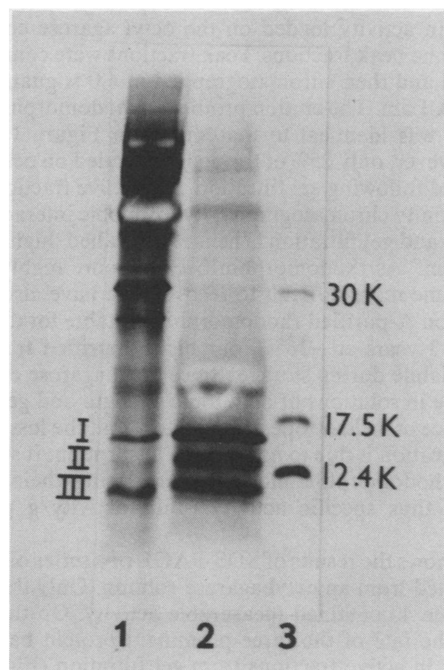

FIG. 2. SDS-PAGE separation of proteins present in active fractions after Con A-affinity chromatography and gel filtration chromatography. Aliquots of Con A-purified rhodomorphin (lane 1) and the pooled active fractions after gel filtration chromatography on Bio-Gel A 1.5m (lane 2), were electrophoresed on a SDS-15% polyacrylamide gel. Lane 3 contained 20 ng each of the marker proteins carbonic anhydrase (30,000), myoglobin (17,500), and Cyt *c* (12,400). The gel was stained for protein by the silver-staining method of Oakley *et al.* (10).

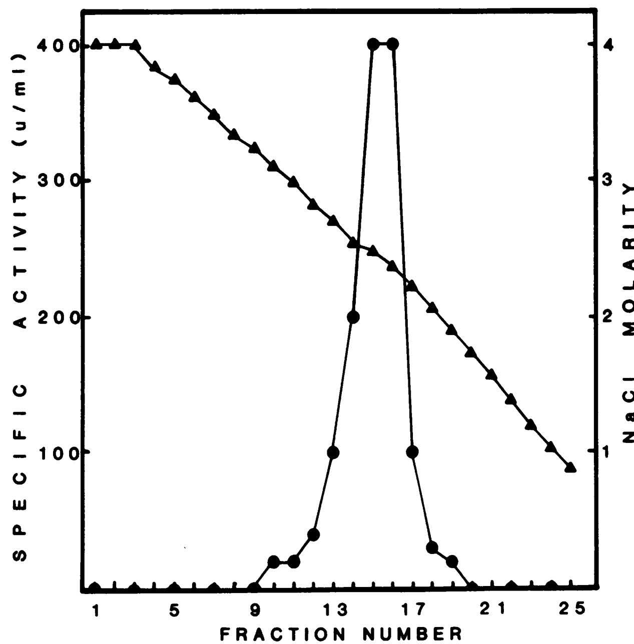

FIG. 3. Hydrophobic interaction chromatography of Con A-purified rhodomorphin. Active fractions after Con A affinity chromatography were made 4.0 M in NaCl and loaded on an octyl agarose column. Fractions were eluted by a 150-ml NaCl linear gradient (4.0–0.4 M) in 20 mM Tris, pH 8.0. The salt concentrations (▲) of the fractions were measured refractometrically and their activity (●) tested.

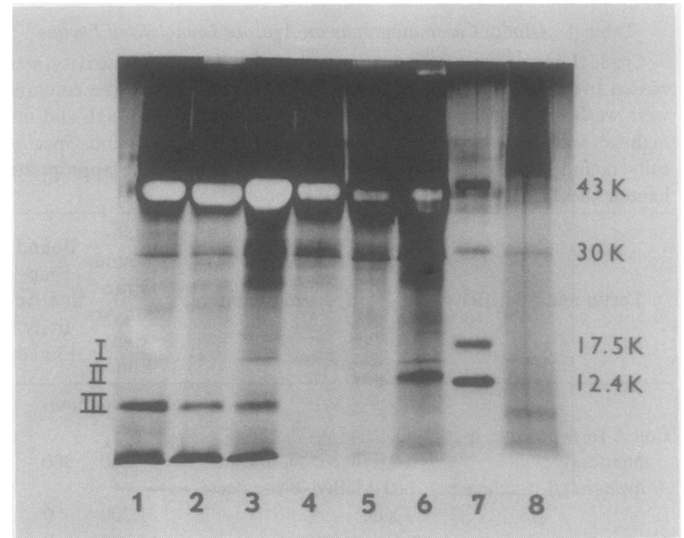

FIG. 4. Comparison by SDS-PAGE of proteins present in fractions separated by hydrophobic interaction chromatography. Aliquots of the fractions eluted from the octyl agarose column (shown in Fig. 3) were loaded in lanes 1 to 6 of a SDS-15% polyacrylamide gel as follows: lane 1, fractions 4 to 6; lane 2, fractions 7 to 9; lane 3, fractions 10 to 12; lane 4, fractions 14 to 16 (activity-containing fractions); lane 5, fractions 18 to 20; and lane 6, fractions 21 to 25. The remainder of fractions 14 to 16 was chromatographed on Bio-Gel A 1.5m in 6 M guanidine HCl; the activity-containing fractions after this step were pooled (1,600 units total) and loaded in lane 8. In lane 7 were loaded 20 ng each of four marker proteins: ovalbumin (43,000), carbonic anhydrase (30,000), myoglobin (17,500), and Cyt *c* (12,400). Proteins were electrophoresed and stained by the method of Oakley *et al.* (10). Bands labeled I, II, and III correspond to the bands seen in Figure 2.

phin preparations were pooled and loaded onto an octyl agarose column. Figure 3 illustrates a typical elution profile for rhodomorphin activity on such a column. Activity is eluted in a single peak at a NaCl concentration of 2.5 M. Typically, 40 to 60% of the rhodomorphin activity loaded on the octyl agarose column was recovered in the peak fractions. Peak fractions were combined and concentrated and then chromatographed in 6.0 M guanidine-HCl on Bio-Gel A 1.5m. The elution profile for rhodomorphin activity, in this case, was identical to that shown in Figure 1 (data not shown). However, only 25% of the activity loaded on octyl agarose was recovered following gel filtration. The active fraction purified by Con A affinity chromatography, hydrophobic interaction chromatography, and gel filtration is henceforth called 'highly purified rhodomorphin.' As rhodomorphin became more highly purified, activity became more difficult to recover. We have also observed that while Con A-purified rhodomorphin is stable for days at 4°C and at least 3 years at −16°C, our highly purified fractions are much more labile during storage. Since octyl agarose chromatography is done in solutions of high ionic strength and gel filtration in the presence of a chaotrope, it is unlikely that the loss of activity during purification is due to nonspecific adsorption. It seems more likely that rhodomorphin molecules are losing their biological activity and thus specific activity (units activity/g protein) is decreasing.

Figure 4 shows the results of SDS-PAGE of a series of combined fractions eluted from an octyl agarose column. Only the fractions loaded in lane 4 contained measurable activity. On this gel, one can follow the fate of the three prominent protein bands which were present in active fractions from gel filtration (Fig. 2). Band III was present in highest concentration in the inactive, high-salt fractions from octyl agarose (Fig. 4, lanes 1–3). Bands I and II were present in highest concentration in the inactive, low-salt fractions (Fig. 4, lanes 5 and 6). Inasmuch as none of these proteins eluted in high concentration in the active fractions on octyl agarose, it appears that none of them is associated with rhodomorphin activity. In fact, there were no bands which were found uniquely in the active fractions from octyl agarose.

Fractions purified by hydrophobic interaction chromatography followed by gel filtration were subjected to SDS-PAGE (Fig. 4, lane 8). As might be expected from the results shown in Figures 2 and 4, the Oakley *et al.* (10) silver stain for protein revealed no unique staining bands. The SDS gel of the active fraction from gel filtration (Fig. 4, lane 8) has a small amount of band III protein as well as some high-mol-wt contaminants. In other experiments, these bands were separated from the active fractions; in these cases, no staining bands were present in SDS gels of highly purified rhodomorphin. Similiar gels were also stained using the Morrisey (9) staining technique for protein: this technique did stain more proteins in lanes containing less-pure rhodomorphin preparations. However, even with this technique, no staining bands could be seen in lanes containing highly purified rhodomorphin fractions. Using protein standards, we found that we could detect one to a few ng protein with both of these techniques. Gels of highly purified rhodomorphin fractions were also stained for carbohydrate by the methods of DuBray and Bezard (4); again, no staining bands could be detected.

Chemical Characterization of Rhodomorphin.

Thermal Stability. Con A-purified rhodomorphin is stable at room temperature for days and for at least 3 years at −16°C. To test the stability of rhodomorphin at higher temperatures, aliquots of Con A-purified hormone solution were treated for 8 min at different temperatures. The results of such an experiment are shown in Table III. Treatment at 40°C reduces activity by one-half and, at 50°C, activity is almost entirely destroyed.

Effect of Proteolytic Enzymes on Rhodomorphin Activity. The lability of rhodomorphin at relatively low temperatures suggested to us that it might be a protein. To test this hypothesis, we measured the effect of the proteinases, trypsin and proteinase K, on rhodomorphin activity. Solutions of Con A-purified rhodo-

Table III. *Thermal Stability of Con A-Purified Rhodomorphin*

One-ml aliquots of Con A-purified rhodomorphin in 0.4 M NaCl, 0.6 mM Tris (pH 8.0) were heated for 8 min at various temperatures. Aliquots were then diluted with f/2-enriched seawater and tested for activity.

Temperature	Activity	Activity Remaining
°C	*units/ml*	*%*
23	400	100
40	200	50
45	100	25
50	20	5
55	15	4

Table IV. *Proteolysis of Rhodomorphin*

Aliquots of Con A-purified rhodomorphin in 2.0 M NaCl, 20 mM Tris (pH 8.0) were incubated at 20°C for 20 h with no proteinase, or with an active or an inhibitor-inactivated proteinase (proteinase K, 0.50 mg/ml; trypsin, 0.25 mg/ml). The proteinases were inactivated by a 1-h incubation with a specific inhibitor, PMSF for proteinase K (at a concentration of 0.5 mg PMSF/mg enzyme) or SBTI for trypsin (at a concentration of 20 mg SBTI/mg enzyme). All samples were exchanged into fresh f/2-enriched seawater on small Bio-Gel P-2 columns prior to their assay for activity.

Proteinase	Time of Addition of Proteinase Inhibitor	Specific Activity
		units/ml
None	None	200
Proteinase K	After proteolysis	0
Trypsin	After proteolysis	80
Proteinase K	Before proteolysis	200
Trypsin	Before proteolysis	200

morphin were incubated with active proteinases. At the end of the incubation, enzyme activity was terminated by the addition of a specific proteinase inhibitor, PMSF for proteinase K or SBTI for trypsin. Before the activity of each solution was tested, the high-mol-wt fraction (mol wt, >1,500) of the solution was buffer-exchanged into f/2. This step removed potentially inhibitory products of proteolytic digestion and unreacted PMSF. To ensure that any decrease in hormone activity was due to the enzymic digestion of rhodomorphin and not to nonspecific binding of rhodomorphin to the enzymes or to the interference by active proteinase with the biological assay, a control was run in which Con A-purified rhodomorphin was incubated with previously inactivated proteinases.

The results of a typical experiment are given in Table IV. Both proteinases reduced the activity of rhodomorphin. Inactivated proteinases did not affect the activity of the hormone. Proteinase K completely abolished activity after 20 h of incubation, Trypsin, at 0.25 mg/ml, reduced activity by 60%. If a higher concentration of trypsin (0.75 mg/ml) was used, rhodomorphin activity could be reduced by 95% (data not shown).

DISCUSSION

The data presented in this paper show that rhodomorphin from *G. pacifica* is a water-soluble molecule which is composed of both protein and carbohydrate (a glycoprotein) and has a mol wt of approximately 14,000. That it contains carbohydrate is shown by its specific binding to Con A. This binding indicates that rhodomorphin contains sterically accessible α-D-mannose and/or α-D-glucose residues. Because rhodomorphin was not bound by the other immobilized lectins tested, it probably does not have L-fucose, *N*-acetyl-glucosamine, and β-D-galactose in sterically accessible positions.

The proteinaceous nature of *G. pacifica* rhodomorphin is shown by its thermal inactivation at relatively low temperatures (40–50°C) and by its inactivation by proteolytic enzymes. The inactivation of rhodomorphin by proteinases also shows that the protein portion of this molecule is accessible to enzymic attack and is essential for biological activity.

The manner in which rhodomorphin binds to and elutes from octyl agarose columns is consistent with its identification as a glycoprotein. The fact that it binds to octyl agarose columns in 4 M NaCl indicates that it contains accessible hydrophobic groups, probably hydrophobic amino acids. Many of the other proteins present in Con A-purified rhodomorphin solutions do not bind even at this high-salt concentration. However, rhodomorphin interacts weakly with octyl agarose and is eluted at a fairly high-salt concentration (2.5 M NaCl). This indicates that it is much more hydrophilic than most proteins, many of which can only be eluted from octyl agarose by organic solvents. The hydrophilic nature of rhodomorphin is probably contributed by the carbohydrate portion of the molecule.

We have been able to separate rhodomorphin from most or all of the other proteins secreted into the medium by *G. pacifica*. At this time, however, we are unable to determine the specific activity (units/g) of purified preparations. The amount of protein and carbohydrate present in active fractions from gel filtration and octyl agarose columns is below the level of sensitivity (<1 μg) of currently available techniques for measuring the concentration of these molecules. Silver stains for proteins on polyacrylamide gels have increased the detection levels to 1 ng protein/band. However, we have not found a staining band which is concentrated in purified active fractions and thus could be attributed to rhodomorphin. The presence of SDS in the polyacrylamide gels precludes the detection of rhodomorphin activity on these gels at this time.

One possible explanation of the lack of a staining band in SDS-polyacrylamide gels of highly purified rhodomorphin preparations is that the 600 to 2,000 units of activity loaded on these gels may be associated with less than a ng of protein and thus be below the level of detection of the stains used. Inasmuch as it is likely that rhodomorphin is losing specific activity as it becomes purer, this is probably an underestimate of the actual amount of rhodomorphin loaded. It is also possible that, for some reason, rhodomorphin does not enter SDS-polyacrylamide gels under the conditions used. Finally, there may be more than 1 ng of rhodomorphin in the gels, but it may not be stained by the protein and carbohydrate stains used. If the first hypothesis is correct, then the concentration of rhodomorphin present in a solution with one unit of activity must be very low, on the order of 10^{-13} to 10^{-14} M.

Rhodomorphin is the first morphogenetic substance to be isolated and purified from red algae. The only other morphogenetic factors to be isolated from any algae are the sexual inducers from members of the colonial green algal genus *Volvox*. The sexual inducer from *V. carteri* is also a glycoprotein but has a mol wt of about 30,000 (6, 14). The sexual inducer in *V. capensis* is an amino acid, L-glutamic acid (15). Rhodomorphin, by inducing the formation of specialized cells which can participate in somatic cell fusion, resembles in function the sexual inducers of other eukaryotic microbes.

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LITERATURE CITED

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