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Immunological Identification of a Centrin Homologue in the Red Alga Gracilaria tikvahiae

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IMMUNOLOGICAL IDENTIFICATION OF A CENTRIN HOMOLOGUE IN THE RED ALGA *GRACILARIA TIKVAHIAE*

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ABSTRACT

Whole cell protein extracts of *Gracilaria tikvahiae* were found to contain antigenically reactive epitopes to the low molecular weight, calcium sensitive protein, centrin. A rabbit antibody specific for a trpE-centrin fusion protein (26/14-1) was used for Westernblot analysis. The centrin homologue was demonstrated to have an relative molecular mass of 20,000 kDa and an acidic isoelectric point of approximately 4.8. In addition, partial purification of the protein using phenyl-sepharose affinity chromatography further substantiates a relatedness between the red algal centrin and centrin reported for other eucaryotes. During the study, alpha tubulin, actin and calmodulin were also identified using immunological probes and Westernblot analysis. Finding centrin in the Rhodophyta supports a highly conserved view of centrin and combined with the results from other cytoskeletal proteins, adds to the growing evidence that red algae are not as "primitive" as previously believed.

IMMUNOLOGICAL IDENTIFICATION OF A CENTRIN HOMOLOGUE IN THE RED ALGA *GRACILARIA TIKVAHIAE*

INTRODUCTION

The red algae, or Rhodophyta, are a cosmopolitan division of protists comprising more than 4,000 species (Kraft 1981). Predominantly marine in nature, they are characterized by floridean starch storage products, non-aggregated thylakoids, phycobilin photosynthetic pigments, and a complex life history lacking any flagellated stage (Gabrielson and Garbary 1986).

Red algae are a problematic division to explain taxonomically, both when considering their intra-ordinal ranking and when trying to explain their relationship to other eucaryotes. Application of cladistic analysis has supported classification as a monophyletic group (Gabrielson et al. 1985), but their relationship to other eucaryotes still remains an enigma. Although relationships to the red algae have been proposed for the Cyanophyta, Cryptophyta and Eumycota (Gabrielson and Garbary 1986), an association with any one of these divisions would not polarize all or even most of the rhodophytan taxonomic characters (Gabrielson et al. 1985).

In order to establish a sister taxon for the Rhodophyta, one must go below the level of morphology or ultrastructure. Most eucaryotic cells exhibit a general conservation of organelles, macromolecules and basic cellular processes. One organelle which has been highly conserved due to its vital function in nucleating and organizing microtubules (MTs) for the mitotic spindle, is the

microtubule organizing center (MTOC) (Pickett-Heaps 1969). An examination and comparison of this organelle at the molecular level should help to elucidate eucaryotic taxonomy. In particular, one protein which seems to have been a highly conserved component of the MTOC in eucaryotes is centrin. This study represents the first report of a centrin homologue in the protistan division, Rhodophyta. The study was undertaken to add to the body of knowledge for centrin distribution in eucaryotes and to relate the significance of this finding to questions of phylogeny.

The MTOC is thought to serve as a cellular focal point for microtubule (MT) nucleation, anchorage and/or stabilization in interphase cells or as a spindle pole in dividing cells (Brinkley 1985). The appearance and cellular address of the MTOC displays variability both between cells of the same division and between cells of different divisions but is most commonly associated with the nucleus. When seen with the electron microscope, it appears as an amorphous electron dense zone from which microtubules diverge. Often the MTOC is found in association with a number of different organelles. There is the centriole-based centrosome of mammalian cells (McIntosh 1983, Mazia 1984), and the nucleus associated organelle (NAO), which may be represented by the spindle pole body (SPB) found in fungi (Heath 1981, Rout and Kilmartin 1990), the predominant "polar ring" (PR) of the red algae (Scott and Broadwater 1990) or any number of different morphologies found in other eucaryotes (Heath 1980). There is also the flagellar basal apparatus (for review see Melkonian 1984) which serves as the principal MTOC

in some unicellular algal cells. The basal body itself directly nucleates the MTs of the flagellar axoneme and immunofluorescence studies have shown that the region surrounding the basal bodies is a focal point for cytoplasmic MTs. During mitosis the basal bodies lose their flagallar axonemes and migrate to the spindle poles as centrioles. The morphology and behavior of these centrioles is apparently the same as those found in animal cells. In addition, there often exists a system of fibrous roots, sometimes called rhizoplasts, extending from and connecting the basal bodies to the nucleus (Pitelka 1974, Melkonian 1980). Portions of these rhizoplasts may serve as the MTOC in interphase and mitotic cells (Stewart et al. 1974), and the entire structure may potentially provide a communicative link between the nucleus and flagella (Kater 1929, Salisbury et al. 1981, Salisbury 1988).

The calcium sensitive nature of the rhizoplast (system II fiber; Melkonian 1980) in the prasinophyte *Tetraselmis subcordiformis* (Salisbury and Floyd 1978) led to the discovery of a novel contractile protein called centrin (Salisbury et al. 1984, Coling and Salisbury 1987). Over 60% of the *Tetraselmis* rhizoplast was found to be composed of centrin. Antibodies raised against this antigen have immunologically recognized similar rhizoplast-like structures in other flagellated cells (Wright et al. 1985, Schulze et al. 1987, Melkonian et al. 1988) and the centrosomes in a diverse set of eucaryotic organisms (Salisbury et al. 1986, Koutoulis et al. 1988, Höfeld et al. 1988, Baron and Salisbury 1988, Hiraoka et al. 1989, Greenwood et al. 1990). Another investigation testifying to

the highly conserved nature of this protein is the stable transfection of algal centrin into a vertebrate quail cell line (QT6) (Salisbury and Greenwood 1990). Preliminary reports suggest that algal centrin transfected into vertebrate cells assembles into a functional centrosome.

Centrin is a low molecular weight (approximately 20,000 M_r) calcium-sensitive contractile phosphoprotein (Salisbury et al. 1984) which has been found associated with 3-8 nm diameter filaments. Two dimensional gel analysis (isoelectric focusing and SDS-Page) of the flagellar roots of *Chlamydomonas* and *Tetraselmis* have revealed that this protein has two closely related acidic isoforms (alpha pI = 4.9 and beta pI = 4.8) (Salisbury et al. 1984, Wright et al. 1985). The more acidic conformation exists as a phosphorylated form corresponding to the extended, noncontracted shape of the associated filament. This form can be rapidly converted to the calcium binding, less acidic contracted isoform by dephosphorylation (Salisbury 1982, Martindale and Salisbury 1990). The contractile-based motility has further been demonstrated to be the result of a twisting and supercoiling of the fibers which make up the striated flagellar roots (Salisbury 1983).

Centrin has also been isolated and sequenced from cDNAs encoding the protein in *Chlamydomonas* (Huang et al. 1988, Salisbury pers. comm.). Sequence comparisons to other calcium binding proteins has demonstrated a close relationship between centrin and members of the EF hand superfamily (Kretsinger 1980). Members of the EF hand superfamily, such as calmodulin, troponin C, and

parvalbumin, are unified by the presence of a series of helix-loop-helix (EF hand) calcium binding domains. The highest sequence homology to centrin was found for calmodulin (45-48%) and, surprisingly, the CDC31 gene product of *Saccharyomyces cerevisiae* (50%) (Huang et al. 1988). A discussion of the significance of these homologies occurs elsewhere in this report.

Another probable relation to centrin is a class of calcium-binding contractile proteins called spasmins (Amos 1971, Amos et al. 1975, Routledge et al. 1976, Routledge 1978). Spasmins are 16 to 20 kDa proteins located in spasmonemes and myonemes of ciliates. These proteins cross react with monoclonal and polyclonal antisera elevated against algal centrin (Melkonian 1989, Bazinet et al. 1990) but much work still remains to be performed at the molecular level to verify the degree of homology between centrin and spasmin.

To date only two physiological roles have been attributed to centrin; however, the possibilities for its involvement in cellular function are exhaustive. Evidence indicates that in the flagellated protist, *Spermatozopsis similis*, centrin is involved in the reorientation of basal bodies during the photophobic response (McFadden et al. 1987). This green alga can redirect its basal bodies from an antiparallel to a parallel arrangement by contraction of the distal connecting fiber (for review of the flagellar basal apparatus see Melkonian 1984). The consequence of this transient reorientation is the conversion of an asymmetrical ciliary wave form to a symmetrical flagellar type beat resulting in a reversal of direction.

There is also evidence that centrin is involved in severing microtubules (Sanders and Salisbury 1989). When viewed in cross section, the flagellar axoneme transition zone of *Chlamydomonas* contains a stellate structure partially composed of centrin. The contraction of centrin may generate torsional and shear forces resulting in microtubule severing and the concomitant excision of the flagella. The incubation of permeabilized *Chlamydomonas* cells with centrin antibodies prevents a calcium induced excision, whereas treatment of cells with calmodulin antibodies does not inhibit the concomitant loss of flagella (Sanders pers. comm.). Centrin may also be involved in other MT severing roles such as the reorganization of MTs into a spindle and directed cell migration (Baron and Salisbury 1988, Sanders and Salisbury 1989, Hollenbeck and Dentler 1991). In fact, the contractile nature of centrin suggests a correlation with Mazia's (1984) "flexible centrosome". Mazia speculated that the centrosome is composed of a chain of MT initiating sites. Such a linear arrangement could take on different conformations which may explain the morphologic diversity of the centrosome in eucaryotes. In addition, the conformational changes of the centrosome may be responsible for the reorganization of MT at various stages of the cell cycle. The contractile nature of centrin seems an ideal candidate to aid in directing these conformational changes via its potential MT severing capability.

Additional roles hypothesized for centrin include: nuclear positioning and shape changes which may indirectly regulate gene expression (Salisbury et al. 1987, Salisbury 1988), basal body

segregation and positioning (Salisbury et al. 1988), and a foundation for cell form and polarity (Salisbury 1989). It will be interesting to discover what role this protein has in the cell dynamics of the red algae.

METHODS

(for buffers and solutions see Appendix A)

Culture. Plants of *Gracilaria tikvahiae* (Forsk.) McLach. were collected in the York river near Yorktown, Virginia and shipped overnight in seawater to Rochester, Minnesota. Plants were then separated, cleaned of visible epiphytes and epifauna and transferred to 1 L flasks containing artificial seawater. Cultures were kept under a 12:12h LD cycle at 18°C until selected for analysis. A rotary shaker was used in the incubator to provide mild agitation (80 r.p.m.).

Sample preparation. A healthy plant was selected, rinsed well in H_2O and rapidly frozen in liquid nitrogen. The frozen tissue was ground to a fine powder with a pre-cooled ceramic mortar and pestle and subsequently weighed. The powder was resuspended 1:1 (w/v) in a 2X SDS sample buffer containing protease inhibitors (2 μg/ml of Pepstatin A, Aprotinin, Chymostatin and Leupeptin (Boehringer Mannheim) and 2.5 mM Phenylmethylsulfonyl flouride [PMSF]). The suspension was extracted by alternate heating (3 min. in a water bath at 90°C) and hand homogenization (Wheaton 15 ml). The homogenate was spun at 21,500g for 10 min. (Beckman J2-21M/E) to remove all large cellular debris. The supernatant fluid was aliquoted into 1 ml microcentrifuge tubes, and enough of a 100% solution of trichloroacetic acid (TCA) was added to bring to a final 10% TCA concentration. TCA extraction occurred for 30 min. on ice

and then tubes were spun at 13,000 r.p.m. for 30 min. The supernatant fluid was discarded and the acid insoluble pellet was washed three times with ethyl ether, resuspended in appropriate buffer and stored frozen at -70°C.

Electrophoresis. For one dimensional electrophoresis (SDS-PAGE), samples were resuspended in 2X SDS sample buffer containing a pyronin Y dye marker, sonicated (5 mm tip Heat Systems-Ultrasonics, Inc. - maximum output) for 5 s., heated for 3 min. at 90°C and loaded onto 0.75 mm vertical acrylamide slab gels (BRL vertical gel electrophoresis system). Sample loads were between 200 and 400 µl. on curtain wells. Polyacrylamide gels consisted of a 3% stacking gel and a 5 - 15% gradient running gel. Gradient gels were cast using a Sage Instruments syringe pump with a limiting solution of 20% acrylamide and a starting solution of 3% acrylamide. Gels were run at 20 mA (with power and voltage unlimited) until the blue dye front reached the stacking interface. Current was reduced to 10 mA for 30 min., then increased to 30 mA until the pyronin Y dye front reached the bottom of the gel. Gels were removed and proteins were either stained with Coomassie Brilliant Blue (CBB) or transferred onto an Immobilon PVDF membrane for Westernblot analysis.

For two dimensional electrophoresis (isoelectric focusing and SDS-PAGE) procedures were followed according to O'Farrell (1975). Samples were resuspended in lysis buffer, briefly sonicated (5 mm tip Heat Systems-Ultrasonics, Inc. - maximum output) and loaded onto tube gels cast in glass tubing (1 mm inside diameter X 15 cm).

The isoelectric gel gradient was from pH 4 to 6. Samples were covered with overlay solution and ß-lactoglobulin A (pI 5.1) and Trypsin inhibitor (pI 4.6) were loaded (3 µg/ml) as isoelectric focusing standards. Markers were not used in gels prepared for transfer due to interference from non-specific binding of the secondary antibody. With power and current unlimited the gels were run at 200 volts (V) for 15 min., increased to 300 V for 30 min., and finally set at 400 V until total volt hours were between 6000 and 8000 V. Tube gels were extruded and incubated at room temperature, with shaking, in 2X SDS sample buffer for a minimum of 30 min. For the second dimension, tube gels were placed on SDS-PAGE vertical gels (3% stacking gel and 15% running gel - BRL) and sealed with 1% agarose. The pattern of running current followed that used for one dimensional SDS-PAGE (above). The gel containing the pI markers was then stained using CBB and three other gels, lacking the markers, were transferred onto Immobilon PVDF membranes.

Protein transfer and Westernblot. SDS-PAGE gels were equilibrated for 15 min. in potassium phosphate transfer buffer (KPTB). Concurrently, Immobilon PVDF membranes were pre-wet with absolute methanol and then equilibrated in KPTB for 15 min. A Westernblot "sandwich" was assembled. A Westernblot "sandwich" was constructed by placing in the following order: a brillo pad, two pieces of blotter paper, the PVDF membrane, the gel, another blotter sheet and brillo pad, between the plastic trasphor casing of a Hoefer Transphor unit. The proteins were transferred from the gel to

membrane in KPTB at 20 V overnight (4°C). Membranes were removed, fixed with fixation buffer (FB) for 45 min., then washed rapidly three times with KPTB and blocked in either nonfat dry milk (NFDM) overnight at 4°C or blocking buffer (BB) for one hr at 37°C. After blocking, membranes were washed three times with wash buffer (WB) (10 min per wash) and incubated in primary antibody diluted in BB for one hour at 37°C. The membranes were again washed three times in WB and subsequently incubated in a peroxidase conjugated secondary antibody (goat anti rabbit or mouse depending on which primary was used) diluted in BB for one hour at 37°C. Membranes were once more washed three times in WB and then reacted with peroxidase mixture. The reaction was stopped by rinsing in H_2O.

The primary antibodies were all affinity purified either by members of the Salisbury lab or by the company from which they were purchased. Other controls were also used to account for the possiblity of non-specific labelling. One control was incubation in pre-immune serum. This control would permit discounting any bands which were the result of a rabbit (primary host) auto-immune response to any *Gracilaria* proteins. Another control was incubation in secondary antibody only. This control allowed for the identification of any bands which may be the result of a goat (secondary host) auto-immune response. Finally, the use of a known source of the protein, such as whole cell extracts from *Chlamydomonas*, allowed for a positive comparison to ensure that the assay was operating correctly.

Column purification. All steps were performed at 4°C unless noted otherwise. Plant thallus was rapidly frozen by dropping into liquid nitrogen and the frozen tissue was transferred to a pre-cooled mortar and pestle and ground to a fine powder. The powder was weighed and then resuspended 1:1 (w/v) with the low calcium extraction buffer (LEB) containing protease inhibitors (2 μg/ml of Pepstatin A, Aprotinin, Chymostatin and Leupeptin (Boehringer Mannheim) and 2.5 mM Phenylmethylsulfonyl flouride [PMSF]). The solution was homogenized by hand (Wheaton 15 ml) and the homogenate spun at 21,500g for 20 min. in a Beckman J2-21M/E ultracentrifuge. The supernatant fluid was brought to 5 mM calcium by addition of 0.1 M calcium chloride stock. The column was prepared by equilibrating phenyl sepharose (Pharmacia CL-4B) with five volumes of high calcium extraction buffer (HEB) and then packing it into a 1 X 8 cm glass column. The column was further equilibrated with 20 ml of HEB passed over the column at 1 ml/min. using an ISCO Wiz peristaltic pump/diluter/dispensor - series 621610004-82342. The extracted high calcium supernatant fluid was then passed over the column at 1 ml/min. The column was rinsed with two volumes of HEB and then eluted with three volumes of LEB. The eluent was dialyzed (Spectropor #1 tubing-MWCO: 6000-8000) for 12 hours against 20 mM ammonium bicarbonate followed by 6 hours against 2 mM ammonium bicarbonate and then finally against H_2O for 3 hours. The dialyzed sample was then shell frozen as one volume and lyophylized (FTS Systems Freeze-Dryer model FD-3-85A-MP).

Lyophylized powder was resuspended in 2X SDS sample buffer and stored frozen at -70°C until needed for gel electrophoresis.

RESULTS

A centrin homologue from *Gracilaria tikvahiae* was identified by Westernblot analysis (Figure 1, lane A). The protein had a relative molecular mass (M_r) of 20,000 kDa which was reactive with an antibody raised in a rabbit against a trpE-centrin fusion protein (26/14-1 diluted 1:1500; secondary antibody diluted 1:1000). Molecular weight estimations were made by comparisons to high and low molecular weight standards (Sigma) and the similar mobility with centrin from *Chlamydomonas reinhardtii* (Figure 1, lane B).

In order to further substantiate this finding, attempts were made to purify the protein. When centrin binds calcium, it exposes a hydrophobic domain which interacts with phenyl sepharose (Gopalakrishna and Anderson 1982). Protein extracts of *G. tikvahiae* containing 5 mM calcium were passed over a phenyl sepharose column. The centrin fraction was collected by eluting with a 1 mM calcium solution. Visualization of this fraction by Westernblot (antibody 26/14-1) yielded a major band around 20,000 M_r with minor banding at lower molecular weights which were not accountable by pre-immune and secondary controls (Figure 1, lane C). It is believed that these lower molecular weight bands may be due to enzymatic digestion by proteases. It is not known what combination of proteases the red algae contain; therefore, a general protease inhibitor "cocktail" was employed. However, this protease inhibitor

mixture proved to be insufficient to eliminate bands presumably caused by protease activity.

In addition, the centrin homologue was found to have an isoelectric focusing point of 4.8 by Westernblot analysis of proteins transferred from two dimensional gels (Figure 2). This acidic pI coincides with that found for centrin in *Chlamydomonas* and *Tetraselmis* (Salisbury et al. 1984, Wright et al. 1985). The isoelectric point of the protein was estimated from its relative mobility to two standards: trypsin inhibitor (pI 4.6) and ß-lactoglobulin A (pI 5.1).

It should be noted that protein biochemistry and attempted immunofluorescence in *G. tikvahiae* proved to be particularly difficult due to the nature of the phycobilisome pigment complexes and cell wall. Phycobiliproteins are photosynthetic accessory pigments which are the major light harvesters of red algae. They comprise between 40 and 60% of all soluble protein (Bennett and Bogorad 1971, Gantt and Lipschultz 1974), are naturally fluorescent throughout a wide absorption spectrum (Gantt 1975), and consist of aggregates of polypeptides with molecular weights between 11,000 and 29,000 kDa (Gantt 1980). All of these properties could affect characterization of the centrin. The preponderance of the pigments in *G. tikvahiae* at 20,000 kDa may have interfered with the resolution of the centrin homologue on polyacrylamide gels. Furthermore, the strong phycobilisome autofluorescence under UV light, produced a high background which could have masked signals from antibody labelling.

The cell wall also obstructed biochemical and immunofluorescence analysis. Cell walls of many red algae consist predominantly of complex sulfated galactans (carrageenans and agars; Gretz and Vollmer 1989); cell walls of *Gracilaria* are known to consist of agar, which is commercially used for culture media and as a thickening agent. These polysaccharides may have complicated protein purification by binding to proteins and hindering separation from other components of the cell. In addition, the cell wall proved difficult to permeabilize for immunofluorescence, using conventional methods. However, recent reports indicate that new immunofluorescence protocols are being successfully applied to red algae (Garbary in press).

Due to the paucity of biochemical information available on cytoskeletal proteins in the red algae (Broadwater et al. in press), other proteins were isolated using SDS-PAGE and Westernblot. However, these findings are not the focus of this study and will not be discussed in greater detail elsewhere. Monoclonal antibodies directed against alpha tubulin [Sigma](Figure 3, lane A), actin [Clone C4/ICN Immunobiologicals](Figure 3, lane B) and calmodulin [1F11 - raised in the Salisbury lab](Figure 3, lane C) were used to probe transfers of whole cell protein extracts. Results indicate the presence of alpha tubulin, actin and calmodulin homologues within *G. tikvahiae* which have similar molecular masses to those proteins found elsewhere in eucaryotes. This result is not surprising considering the highly conserved nature of these proteins in other eucaryotic lines.

DISCUSSION

Many consider the Rhodophyta to represent "one of the oldest groups of eucaryotic algae" (Lee 1989), and sequence analysis of 5S ribosomal RNA from *Batrachospermum* and *Porphyra* suggests their divergence from the eucaryotic lineage 1.2 billion years ago (Hori and Osawa 1987) However, recent studies utilizing sequence comparisons of both the small 16S-like rRNA from *Gracilaria lemaneiformis* (Bhattacharya et al. 1990) and the large 28S cytoplasmic rRNA from *Porphyridium purpureum* (Perasso et al. 1989), indicate a narrow period of radiation for higher plants, fungi, animals and most protists, including the red algae (Bhattacharya et al. 1990). The discovery of the highly conserved centrin homologue within this group lends support either to the notion of a major eucaryotic radiation or to the possibility that centrin has a very long evolutionary history. Sequence comparisons among the different eucaryotic centrins should help elucidate these phylogenetic relationships.

A characteristic posing particular problems in establishing a sister taxon for the red algae is the lack of a flagellated stage or evidence for any residual structures such as centrioles/basal bodies in any phase of the rhodophytan life history. The problem is especially apparent when one considers that almost all other

eucaryotic groups possess a flagellar apparatus (FA), with the exception of the Basidiomycetes, Ascomycetes and higher plants.

The lack of this feature also raises a question about the character of the ancestral red algae. Was the primitive red algal cell flagellated or do flagella represent a derived trait in other eucaryotes? The non-flagellated state of the higher fungi and plants may represent an adaptation to a terrestrial environment, but the red algae are aquatic and would have presumably benefited from retaining flagella. Nevertheless, the present survival of the red algae signify that flagella may not necessarily be an adaptive advantage for this division. However, if one considers the position of Stewart and Mattox (1980), that the FA came before the mitotic spindle and that the spindle evolved from the FA, one would have to conclude that, indeed, primitive red algae must have had flagella. Centrin was initially characterized and localized as a basal body associated protein (Salisbury and Floyd 1978, Salisbury et al. 1984). Although there is no direct evidence that this relationship has always occurred, the discovery of centrin in red algae is tentatively suggestive for a flagellated, red algal ancestor. Perhaps a better way to determine the presence of a past flagellated state would be to probe a genomic library of a red algal species for a specifically related flagellar gene such as one from the uni-linkage group of *Chlamydomonas* (Dutcher 1989).

In conjunction with the evolution of the mitotic spindle, Stewart and Mattox (1980) also postulated that the spindle-

organizing rhizoplast of some flagellated protists (*Chlamydomonas*, Kater 1929; *Ochromonas*, Bouck and Brown 1973; *Tetraselmis*, Stewart et al. 1974) has been altered in other eucaryotic organisms. If this hypothesis is true, then the NAO or polar ring region of the red algae may represent a vestigial rhizoplast. In fact, what appears to be a rhizoplast-like structure can be observed extending from the NAO region in some prophase cells of coralline red algae and several other species (Scott pers. comm.). Centrin's association with rhizoplasts (Salisbury et al. 1984, Wright et al. 1985, Schulze et al. 1987, Melkonian et al. 1988) and centrosomes (Salisbury et al. 1986, Koutoulis et al. 1988, Höfeld et al. 1988, Baron and Salisbury 1988, Hiraoka et al. 1989, Greenwood et al. 1990) in other eucaryotes, and the identification of centrin in *Gracilaria tikvahiae* is suggestive and possibly supportive of the Stewart and Mattox hypothesis. Nevertheless, such predictions should be made with extreme caution, particularly in light of the need for more information about centrin's physiological role, the lack of details about centrin's origin and the absence of information about centrin's localization within the red algae (studies in progress).

One would expect the localization of centrin to be at the MTOC or centrosome (NAO-region) of *Gracilaria tikvahiae*. In many other observations to date, centrin epitopes have localized to the centrosome (Wright et al. 1985, Salisbury et al. 1986, Schulze et al. 1987, Melkonian et al. 1988, Koutoulis et al. 1988, Höfeld et al. 1988, Baron and Salisbury 1988, Hiraoka et al. 1989, Greenwood et

al. 1990). In most eucaryotic cells the MTOC serves as a MT focal point in both interphase and mitosis. However, according to electron microscopical studies, cytoplasmic MTs do not appear to be very prevalent in red algae. There are, however numerous reports for the existence and involvment of MTs in the spindle of dividing cells (Broadwater et al. in press). During mitosis, spindle MTs appear to originate from an electron dense zone associated with the NAO but do not actually come in contact with the NAO. This type of MT distribution coincides with that typically seen in centriolar-based centrosomes (Brinkley 1985). This affiliation suggests that the NAO, and possibly centrioles or other MTOC-associated organelles in other species, may be utilizing the spindle as a means of ensuring their existence in progeny cells (Heath 1980). It seems likely that centrin may be involved in this segregation and/or equipartitioning of the red algal centrosome or NAO.

As with centrosomal structures in many other eucaryotic cells, the red algal NAOs with polar ring morphology undergo a semi-conservative style of replication (Scott and Broadwater 1990). This type of duplication necessitates a template biogenesis and a movement of the polar rings during mitosis to the spindle poles of dividing cells (Scott and Broadwater 1990). Similar behavior occurs in spindle pole bodies (SPBs) of Ascomycetes and Basidiomycetes (Heath 1981). In addition, both red algae and higher fungi lack any centriole or basal body complex (Rout and Kilmartin 1990). Recent cloning of the centrin gene from *Chlamydomonas* has demonstrated a significant sequence homology between centrin and CDC31 gene

product, presumed to localize to the SPB of *Saccharomyces cerevisiae* (Baum et al. 1986, Huang et al. 1988). Temperature sensitive mutants for the CDC31 gene in *Saccharomyces cerevisiae* are characterized by a
single large SPB instead of two SPBs (Baum et al. 1986). This phenotype may be the result of an arrest of the duplication process or interference in the segregation of the duplicated SPBs. In either case, there is an implied involvement of a centrin homologue.

It was partially because of the similarity between yeast and red algal NAOs that this study was undertaken. However, identification of a centrin homologue in red algae lacks the necessary information to make a direct comparison to the yeast SPB because of differences in their characterization. Protein sequence analysis and perhaps demonstration of cross reactivity of the yeast SPB with the antibody used in this study may help to elucidate the possible similarities and relationships between these two NAOs.

Another potential role for centrin in the red algae concerns the movement of the nucleus and pyrenoid to the cell periphery observed during telophase in the unicellular red algae *Rhodella violacea* and *Rhodella maculata* (Patrone et al. in preparation). The contractile nature of centrin has been shown to play a role in a variety of cell motility phenomenon (Wright et al. 1985, Salisbury et al. 1987, McFadden et al. 1987, Salisbury et al. 1988, Koutoulis et al. 1988). It could very well retain this capability in the Rhodophyta.

Additionally, centrin may possibly be involved in gene regulation. Previous studies have suggested the possible involvement of centrin in turning on tubulin genes after flagellar excision (Salisbury et al. 1987, Salisbury 1988). Given the transient nature of the rhizoplast-like structure sometimes seen at the prophase spindle poles in coralline red algae, the paucity of cytoplasmic microtubules which could act as a tubulin pool (see Broadwater et al. in press), and assuming the presumed rhizoplast contains centrin, there may be a connection to tubulin induction for the synthesis of the mitotic spindle in actively dividing red algal cells.

As more of the molecular nature and assembly properties of the various centrins are revealed, and techniques improve for working on red algal systems, we can look forward to discovering the function and position of centrin homologues in the Rhodophyta. Sequence comparisons between red algal centrin and other eucaryotes should also help clarify the phylogenetic relationships and divergence of the various divisions. For now, the presence of centrin is a new piece of evidence that can be added to the growing information from rRNA sequence analysis, mitotic cell morphology (Heath 1986), and the apparently conserved nature of red algal proteins such as tubulin, actin, calmodulin, that red algae are not a "primitive" eucaryotic division.

APPENDIX A

BUFFERS AND SOLUTIONS

All analytical grade chemicals from Sigma unless noted otherwise.

Culture:

A. Artificial seawater: 3.5% (w/v) "Marine Mix" artificial seawater crystals, and 10 mM Hepes buffer, pH to 7.6. [store at18°C in incubator]

Electrophoresis:

A. 2X SDS sample buffer: 0.125 M Tris base pH 6.8, 4% (w/v) sodium dodecyl sulfate (BioRad), 2 mM ethylenediamine-tetra-acetate (EDTA) pH 6.8, 20% (v/v) glycerin, 10% (v/v) ß-Mercaptoethanol, and 0.6 mg/ml bromophenol blue (BioRad). [store frozen at -20°C]

B. 30% acrylamide stock: 29.2% (w/v) acrylamide (BioRad)and 0.8% (w/v) bisacrylamide (BioRad). [store in dark at 4°C]

C. Running gel buffer: 1.5 M Tris base, 0.8% (w/v) SDS (BioRad) and 0.04% (v/v) sodium azide (NaN_3), pH to 8.8. [store at 4°C]

D. Stacking gel buffer: 0.5 M Tris base, 0.8% (w/v) SDS (BioRad) and 0.04% (v/v) NaN_3, pH to 6.8. [store at 4°C]

E. Monomer solution: 38% (w/v) acrylamide (BioRad) and 2% (w/v) bisacrylamide (BioRad) [store in dark at 4°C]

F. Isoelectric focusing gel solution: 5.5 g Urea, 2.0 ml of 10% Nonidet P-40, 1.0 ml of monomer solution, 0.4 ml ampholines (BioRad pH 4 - 6), 0.1 ml ampholines (BioRad pH 3 - 10) and 2.5 ml H_2O, degas solution for 10 min. then add 10 µl of N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and 10 µl of fresh 10% ammonium persulfate.

G. Lysis buffer: 5.5 g Urea, 2.0 ml of 10% Nonidet P-40 solution, 0.4 ml ampholines (BioRad pH 4 - 6), 0.1 ml ampholines (BioRad pH 3 - 10), 0.5 ml ß-Mercaptoethanol, 1.0 ml glycerin, and 2.0 ml H_2O. [divide into 1 ml aliquots and freeze at -70°C]

H. Sample overlay solution: 5.5 g Urea, 0.4 ml ampholines (BioRad pH 4 - 6), 0.1 ml ampholines (BioRad pH 3 - 10), and 5.5 ml of H_2O. [divide into 1 ml aliquots and freeze at -70°C]

I. 10X reservoir buffer (BRL): 0.25 M Tris base, 1.92 M glycine, and 0.04% (v/v) NaN_3, pH to 8.8. [store at room temperature]

J. Coomassie Brilliant Blue stain (CBB): 0.1% (w/v) Coomassie Brilliant Blue (BioRad), 50% (v/v) methanol, and 7% (v/v) glacial acetic acid. [store at room temperature]

K. CBB destain: 10% (v/v) methanol, and 7% (v/v) glacial acetic acid. [store at room temperature]

Protein transfer and Westernblot.

A: 25 mM Potassium phosphate transfer buffer (KPTB): 25 mM potassium phosphate-monobasic (KH_2PO_4) approx. pH 4.6, 25 mM potassium phosphate-dibasic (K_2HPO_4) approx. pH 8.0, add KH_2PO_4 to K_2HPO_4 to bring pH to 7.0. [store at room temperature]

B. Tris buffered saline (TBS 1X stock): 10 mM Tris, 150 mM sodium chloride (NaCl) pH to 7.4. [store at room temperature]

C. Fixation buffer (FB): 0.2% glutaraldehyde (EMS), bring to volume (Q.S.) with KPTB, pH to 7.0. [make fresh for each use]

D. Blocking buffer (BB): 2.0% bovine serum albumin (BSA), 10.0% normal goat serum, 0.05% Tween-20, Q.S. with TBS (1X stock) pH to 7.4. [store at -20°C]

E. Nonfat dry milk buffer (NFDM): 5.0% nonfat dry milk (Carnation), 0.04% NaN_3, Q.S. with TBS (1X stock), pH to 7.4. [store at 4°C]

F. Wash buffer (WB): 0.05% Tween-20, Q.S. with TBS (1X stock), pH to 7.4. [make fresh for each use]

G. Peroxidase reaction mix: (each solution may be made in advance and stored at 4°C) Prior to each reaction the solutions should be mixed in the following proportions: 0.1 ml of solution **a** + 0.2 ml of solution **b**. + 0.1 ml of solution **c**. + 9.6 ml of H_20

solution **a**: 1.7% (w/v) 3-amino-9-ethylcarbazole , Q.S. with dimethylformamide (DMSO).

solution **b**: 2.35% (w/v) succinic acid, 11.9% (w/v) sodium acetate, 0.4% (w/v) thimerasol.

solution **c**: 3.0% (v/v) hydrogen peroxide.

Column protein purification.

A. High calcium extraction buffer (HEB): 0.5 M potassium chloride (KCl), 20 mM Tris base, and 5 mM calcium chloride ($CaCl_2$), pH to 6.8. [store at 4°C]

B. Low calcium extraction buffer (LEB): 0.5 M potassium chloride (KCl), 20 mM Tris base, and 1 mM calcium chloride ($CaCl_2$), pH to 6.8. [store at 4°C]

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FIGURE 1

One dimensional Westernblot using polyclonal anti-centrin antibody (26/14-1).

Lane A: TCA precipitate from whole cell extract of *Gracilaria tikvahiae*.

Lane B: Whole cell extract of *Chlamydomonas reinhardtii*.

Lane C: Phenyl-sepharose purified protein from *Gracilaria tikvahiae*.

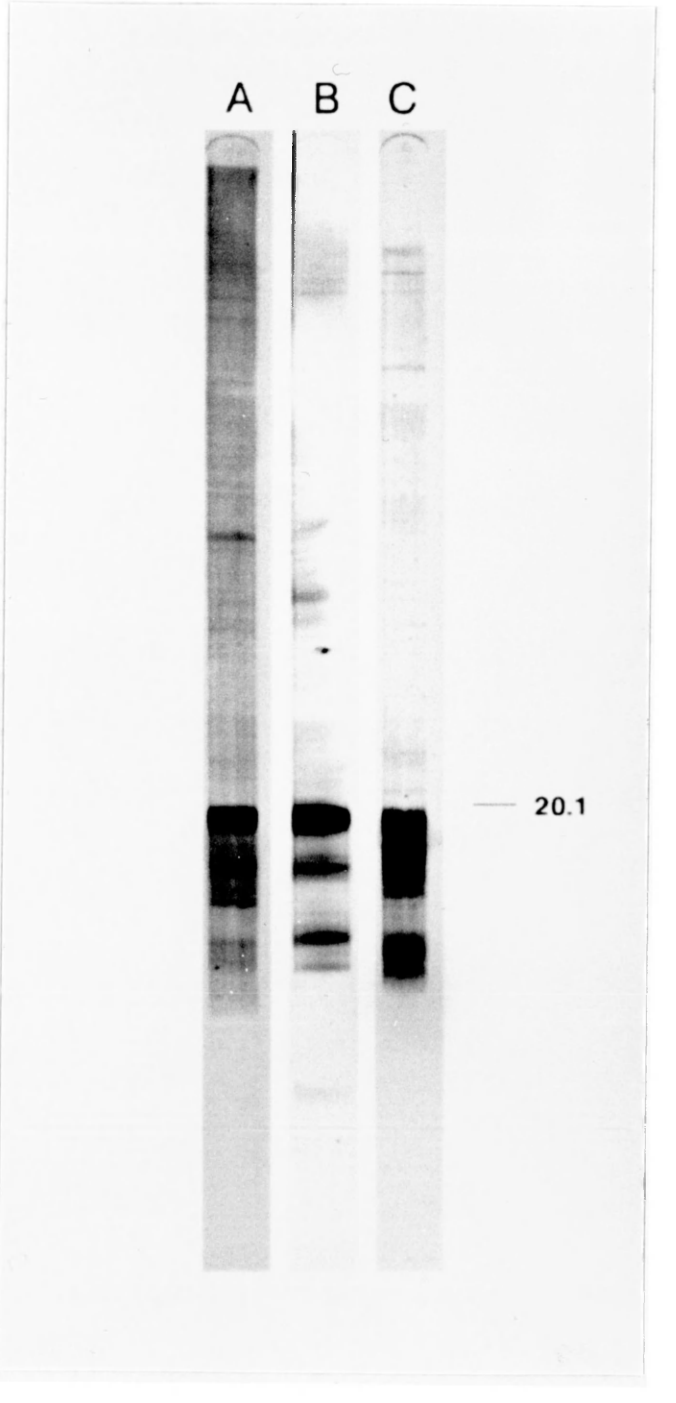

A
B
C
20.1

FIGURE 2

Two dimensional Westernblot using polyclonal anti-centrin antibody (26/14-1). The antibody recognized a protein with an approximate isoelectric point of 4.8 (arrow)

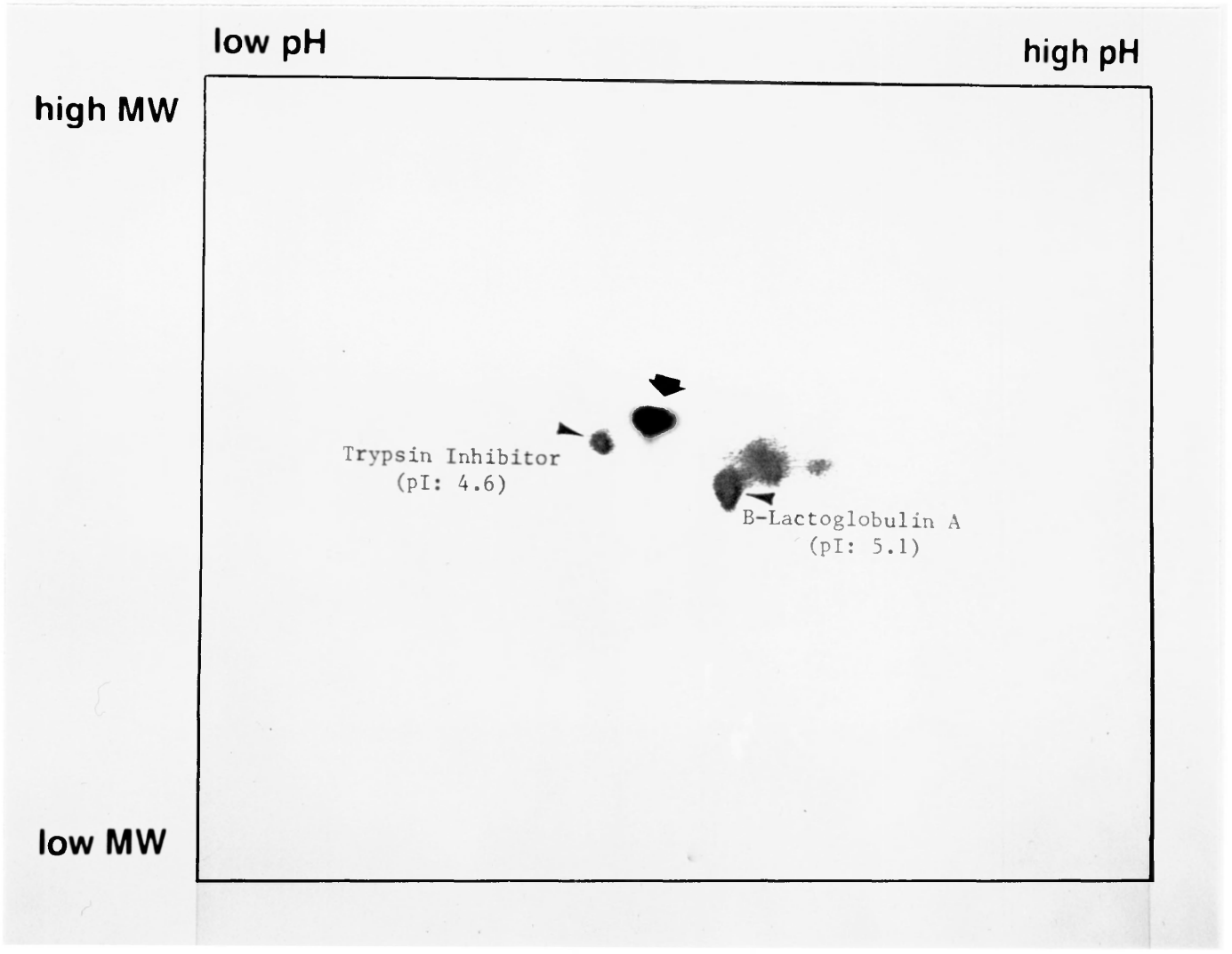

low pH
high pH
high MW
low MW
Trypsin Inhibitor
(pI: 4.6)
B-Lactoglobulin A
(pI: 5.1)

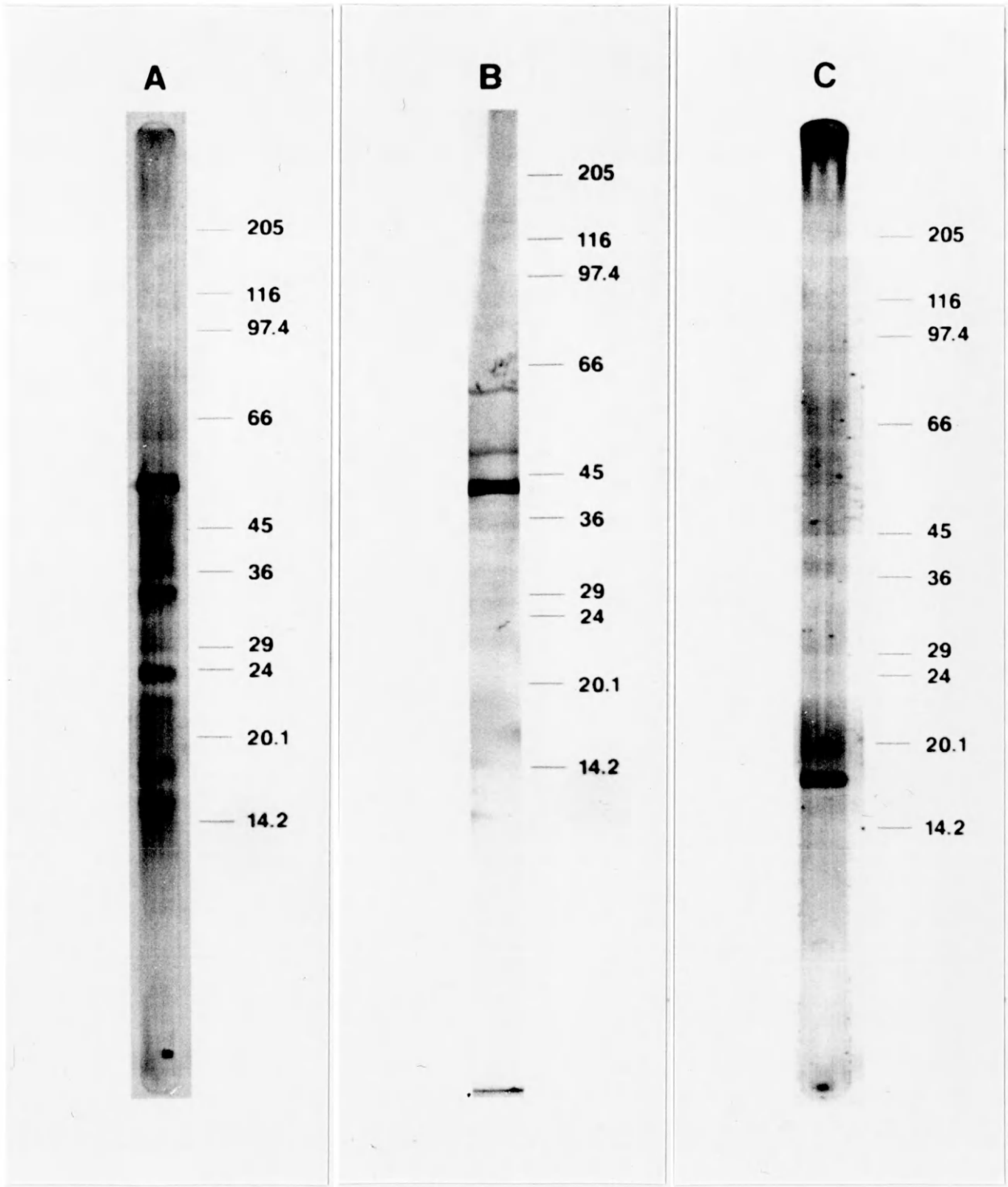
FIGURE 3

One dimensional Westernblot of whole cell extracts of *Gracilaria tikvahiae*.

Lane A: Monoclonal anti-alpha tubulin antibody (Sigma) recognizes a protein of 53,000 M_r.

Lane B: Monoclonal anti-actin antibody (Clone C4/ICN ImmunoBiologicals) recognizes a protein of 43,000 M_r.

Lane C: Monoclonal anti-calmodulin antibody (1F11 raised in the laboratory of J. L. Salisbury) recognizes a protein of 18,000 M_r.


A
205
116
97.4
66
45
36
29
24
20.1
14.2
B
205
116
97.4
66
45
36
29
24
20.1
14.2
C
205
116
97.4
66
45
36
29
24
20.1
14.2

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