

Analysis of Proteinaceous Components of the Organic Matrix of Endoskeletal Sclerites from the Alcyonarian *Lobophytum crassum*

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Abstract. The mesoglea of alcyonarians is occupied by an abundance of minute calcitic sclerites. The sclerites of the alcyonarian *Lobophytum crassum* contain a water-soluble organic matrix comprising 0.48% of the sclerite weight and a water-insoluble fraction comprising 1.15% of the sclerite weight. Analysis of proteinaceous components in the soluble fraction shows a particularly high content of aspartic acid, followed by alanine, glycine, and glutamate. Aspartic acid, glycine, alanine, and glutamate are the most abundant residues in the insoluble fraction. In both cases, the fractions show the highest concentration of aspartic acid from the total proteins. In an *in vitro* assay, we show that the matrix proteins extracted from the calcitic sclerites induce the formation of amorphous calcium carbonate prior to its transformation into the calcitic crystalline form. We also show scanning electron micrographs of the rhombohedral calcite crystals used as template, the protein imprinted with these crystals. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of both matrices shows the protein fractions at 67 and 48 kDa. The soluble matrix shows two additional faint bands. Both fractions stain for a carbohydrate at 67 kDa, indicating a glycoprotein at this molecular weight. A newly derived protein sequence was subjected to bioinformatics analysis involving identification of similarities to other acidic proteins. The identification of these proteins in alcyonarian endoskeletal sclerites emphasizes the fundamental importance of such acidic proteins and sheds more light on the functions of these proteins in the processes of biocalcification.

Key words: Alcyonarian — Calcification — Organic matrix — Protein — Sclerite

Decalcification of sclerites in alcyonarians reveals an organic matrix pattern which is closely related to the size and orientation of the crystals [1]. The organic matrix is repeatedly implicated as a major controlling factor in calcification [2, 3]. Among the invertebrates, the majority of organic matrix analyses have been conducted on

mollusks [4–6]. Approximately 0.01–10% of the weight of mollusk shells is composed of the organic matrix [7]. This matrix consists mostly of proteins and carbohydrates and is composed of water-soluble and water-insoluble components. One of the most remarkable attributes of the calcified tissues formed by many different phyla is that they contain a most unusual assemblage of proteins and glycoproteins that are very acidic [8]. The precise function of these proteins and the mechanisms that operate to control their deposition as the biocrystal grows are of great interest but are largely unknown. The calcified proteins in a number of groups of organisms have been studied in some detail, chemically and microscopically, in order to elucidate the nature of the mineral-matrix relationship [9]. Because of the potential influence of matrix proteins on the calcification process, it is informative to compare their amino acid compositions. In this way, similarities or differences within the same group or among various groups can be detected. Patterns identified by these analyses may provide clues to the calcification process.

A comparison of the calcified and noncalcified tubes of polychaete worms illustrates the potential significance of protein composition to calcification [10]. Calcified serpulid worm tubes have a protein which is high in the acidic amino acids, with aspartic acid in the greatest abundance. In contrast, the noncalcified, cemented tubes of sabellariid worms have a protein composition very high in glycine and serine. Glycine is also commonly abundant in noncalcified molluscan periostracum [11].

Recent attention has focused on the protein composition of skeletal structures of corals [12–15]. Corals, one of the major reef-forming groups of invertebrates, are conveniently divided into two major classes: scleractinian and alcyonarian. Scleractinian corals secrete the massive aragonite skeletons, which are major reef-formers in tropical waters, while alcyonarians contain very ornate, discrete, high magnesium-calcite sclerites embedded in the fleshy tissue [13]. The composition of the calcified protein of these two groups provides an

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interesting comparison because of the diverse nature of the skeletal features. In spite of the relatively large amount of information available concerning molluscan and other invertebrate matrices [6, 16, 17], very little is known regarding the matrix components of alcyonarians. Young [14] and Mitterer [9] have reported the amino acid composition of calcified structures of scleractinian and alcyonarian corals; however, no distinction has been made between the soluble and insoluble matrices. It is very much essential to understand in detail the fraction of both in alcyonarians as the matrices play a key role in the calcification processes by determining the nature of the minerals [18] as well as the general morphology of the biomineralization formed. In the present study, the organic matrix from the sclerites of the alcyonarian *Lobophytum crassum* has been analyzed for its amino acid content and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in order to obtain further information concerning sclerite formation and general mechanisms of biocalcification.

Materials and Methods

Separation of Sclerites

Sclerites were separated from the coral colony (*L. crassum* Marenzeller) by mechanical and chemical treatments as follows. The coral colony was cut using sharp scissors into small pieces. The pieces were ground five or six times with a mixer machine (BM-FE08; Zqjirushi, Kyoto, Japan) and washed with tap water until the sclerites were obtained. The collected sclerites were stirred vigorously in 1 M NaOH for 2 hours and subsequently in 1% NaClO solution for 2 hours to remove the fleshy tissues and debris. Treated samples were washed under tap water until the sclerites were completely cleaned. Finally, samples were washed with distilled water (five times) to remove unwanted substances.

Preparation of Organic Matrix

Decalcification. The mechanically and chemically cleaned sclerites were extensively washed in distilled water and decalcified in 0.5 M ethylenediaminetetraacetic acid (pH 7.8) overnight. The decalcifying solution was centrifuged (H-103 Kokusan, Kyoto, Japan) at 4,000 rpm (15 minutes), and the soluble matrix in the supernatant and the insoluble matrix in the precipitate were subsequently lyophilized.

Fractionation. Filtrated samples were passed through two tandemly connected Sep-Pak C₁₈ cartridges (Waters Associates, Milford, MA) to separate the soluble macromolecules, followed by passing 10% acetonitrile (2 mL/three times). Finally, the absorbed macromolecules were eluted in 50% acetonitrile (2 mL/three times), then frozen in a deep freezer and concentrated by drying under a vacuum.

Amino Acid Analysis

The soluble and insoluble fractions were hydrolyzed under reduced pressure in 200 μ L/0.2 mL of 6 N HCl at 110°C for 22 hours after flashing twice with nitrogen. The samples were

analyzed using a Hitachi (Tokyo, Japan) L-8500 amino acid analyzer. The amount of protein in each sample was determined from the molar yields of amino acids.

Gel Electrophoresis

The dried sample was dissolved in 50 μ L of sample buffer (6.25 M Tris-HCl [pH 6.8], 50% glycerol, 10% SDS, 5% 2-mercaptoethanol, 0.5% bromophenol blue [BPB]) and heated at 100°C for 3 min. It was then electrophoresed (about 2 hours) with SDS-PAGE according to the method of Laemmli [19] in 1.5 M Tris-HCl (pH 8.8), 0.5 M Tris-HCl (pH 6.8), 10% SDS, acrylamide-bis, 10% ammonium persulfate, and TEMED. Prestained SDS-PAGE standards (protein marker) were used for electrophoresis, which contained phosphorylase B (113 kDa), bovine serum albumin (92 kDa), ovalbumin (52.9 kDa), carbonic anhydrase (35.4 kDa), soybean trypsin inhibitor (29 kDa), and lysozyme (21.5 kDa). After finishing the electrophoresis, the gel was stained by Coomassie brilliant blue R-250 (CBB, 1% in 40% Me-OH, 7.5% acetic acid) and destained (10% Me-OH, 7.5% acetic acid) to visualize the protein bands. Carbohydrates were detected following the method described by Zacharius et al. [20] and using the Schiff reagent according to Segrest and Jackson [21].

Amino Acid Sequences

For the preparation of the amino acid sequence analysis, the transferred visualized bands on polyvinylidene difluoride (PVDF) membrane were cut [13], and sequencing was carried out by a protein sequencer (model PPSQ-21A; Shimadzu, Kyoto, Japan) for 24 hours.

Scanning Electron Microscopy

To examine the crystal form, crystals were grown from CaCl₂ (20 mM) and NaHCO₃ (20 mM) at pH 8.7 (approximately) on the purified protein sheet; soluble polyanionic proteins or bovine serum albumin (control) were added to a final concentration of 6 μ g mL⁻¹. All samples were placed in a sample holder, dried for a few days, and coated with palladium gold using an ion coater (Eiko, IB-1, JEOL, Tokyo, Japan). Finally, the samples were examined under a scanning electron microscope (JSM-6060, JSM-6060LV, JEOL, Tokyo, Japan) operated at 20 kV.

Results

The soluble matrix comprised 0.48% of the sclerite weight and emerged as a white fluffy material. When lyophilized, the insoluble matrix emerged as a crumbly white-brown material comprising 1.15% of the sclerite weight. The amino acid composition of the soluble and insoluble organic matrix fractions is presented Tables 1 and 2. The composition of the soluble matrix was characterized by a predominance of aspartic acid, comprising greater than 37% of all residues. Next in abundance was alanine, making up approximately 14%, followed by glycine at about 11%, then glutamate at about 8%. The total acidic residues (asp + glu) comprised almost half of the protein. The neutral amino acids (gly, ala, val, leu, and isoleu) were less abundant, with values of 2–11 mol%. Each of the remaining amino

Table 1. Amino acid compositions of the protein in the total soluble fraction separated by the Sep-Pak C₁₈ cartridge from the endoskeletal sclerites of *L. crassum*

Amino acid	Molecular weight of residues	Soluble fraction	
		nmol	mol%
Cystine acid	151.14	0.1267	0.44
Aspartic acid	115.09	10.7382	37.56
Threonine	101.10	1.1921	4.17
Serine	87.08	1.0461	3.66
Glutamic acid	129.11	2.2531	7.88
Glycine	57.05	2.9203	10.21
Alanine	71.08	3.8823	13.58
Valine	99.13	1.0807	3.78
1/2-Cystine	103.14	0.4397	1.54
Methionine	131.20	0.1697	0.59
Isoleucine	113.16	0.6398	2.24
Leucine	113.16	0.7415	2.59
Tyrosine	163.17	0.1874	0.66
Phenylalanine	147.17	0.4116	1.44
Lysine	128.17	0.6821	2.39
Histidine	137.14	0.2302	0.81
Arginine	156.19	0.4675	1.64
Tryptophan	186.20	0.0000	0.00
Proline	97.11	1.3836	4.84
Protein total			100.00

Table 2. Amino acid compositions of the protein in the total insoluble fraction from the endoskeletal sclerites of *L. crassum*

Amino acid	Res. MW.	Insoluble fraction	
		nmol	mol%
Cystine acid	151.14	0.5367	1.65
Aspartic acid	115.09	5.6115	17.25
Threonine	101.10	1.8996	5.84
Serine	87.08	2.4691	7.59
Glutamic acid	129.11	2.6018	8.00
Glycine	57.05	5.1840	15.94
Alanine	71.08	2.6268	8.08
Valine	99.13	1.4164	4.35
1/2-Cystine	103.14	0.8915	2.74
Methionine	131.20	0.1718	0.53
Isoleucine	113.16	1.1053	3.40
Leucine	113.16	1.4976	4.60
Tyrosine	163.17	0.2921	0.90
Phenylalanine	147.17	0.8396	2.58
Lysine	128.17	1.3657	4.20
Histidine	137.14	0.5861	1.80
Arginine	156.19	1.3390	4.12
Tryptophan	186.20	0.0000	0.00
Proline	97.11	2.0890	6.42
Protein total			100.00

acid residues occurred at approximately 0.9% or less of the total residues.

In contrast, the insoluble matrix of sclerites was again most abundant in aspartic acid but comprised only 17% (approximately) of this fraction. Also at about 16% of all residues was glycine, followed by alanine and glutamate at about 8%, then serine at approximately 7%. The

remaining amino acid residues were fairly evenly distributed, each occurring at about 5% or less of the total residues. In both fractions, tyrosine occurred an order of magnitude lower than the next lowest occurring residue and tryptophan was absent (0 mol%). The acidic residues comprised approximately 46% and 25% of the soluble and insoluble matrices, respectively. The basic residues comprised about 5% and 10% of the soluble and insoluble matrices, respectively. Aromatic residues accounted for 2% of the soluble matrix and 4% of the insoluble matrix, whereas tryptophan was absent in both cases.

The results of SDS-PAGE analysis are presented in Figure 1. Protein bands were reproducible by CBB and silver staining but variable in intensity from gel to gel. As seen in Figure 1, proteins of both the soluble and insoluble fractions stained at a molecular weight of approximately 67 and 48 kDa. In addition, two faint bands were observed in the soluble fraction at 102 and 37 kDa. The molecular weights of standards are shown in Figure 1. After electrophoresis, both soluble and insoluble matrix proteins were transferred onto PVDF membranes in a liquid electroblotter (Mini Trans Blot[®]; Bio-Rad, Richmond, CA) and stained for a carbohydrate with periodic acid-Schiff (PAS) at a molecular weight of approximately 67 kDa (Fig. 2, lanes 1, 2). Glycoproteins appeared as red/purple bands on the membranes. The molecular weights of standards are shown in Figure 2.

The matrix proteins were further purified by electroelution treatment as previously reported [13]. Purified proteins were then transferred to a PVDF membrane according to the Western blotting method. Out of four proteins on PVDF membranes, three (except for the 37 kDa protein) were distinct and selected for sequence analysis [13]. To determine the amino acid sequences of these proteins, we attempted to sequence the N terminus. In the present study, we discuss only the sequencing of the 48 kDa protein because it contained a higher abundance of acidic amino acids than the 102 kDa protein (data not shown) and information for the 67 kDa protein has already been reported (see Rahman and Isa [13] for review). The result of the 48 kDa protein sequence is as follows:

NH₂-GEVVFELKEEEIKDGFGEFA—COOH.

A bioinformatics analysis of the 48 kDa protein was subjected to sequence alignment with another organism's protein. Alignment of this sequence with others using the WU-BLASTP program, version 2.0MP-WashU (accessed Dec. 5, 2004), against the Swiss-Prot protein database showed significant sequence similarity (EMBL-EBI (European Bioinformatic Institute) Dr. Warren Gish) (Fig. 3). In total, the best seven matched proteins were selected for this sequence alignment. The description of aligned proteins is as follows: (1) probable ribokinase (EC

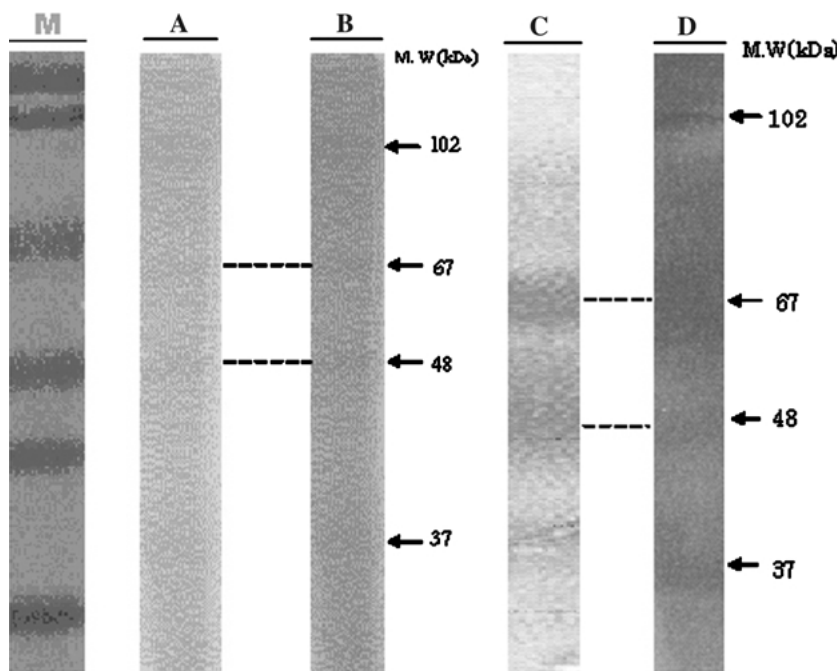


Fig. 1. SDS-PAGE of the proteins from the sclerites of *L. crassum*. Silver-stained SDS-PAGE separation of (A) the total assemblage of insoluble matrix proteins and (B) the total assemblage of soluble matrix proteins. Gels C and D are stained for proteins with CBB for insoluble and soluble matrix fractions, respectively. *Arrows* indicate significant protein bands. *Broken lines* indicate the same proteins in the soluble and insoluble fractions. M, protein marker; MW, molecular weight.

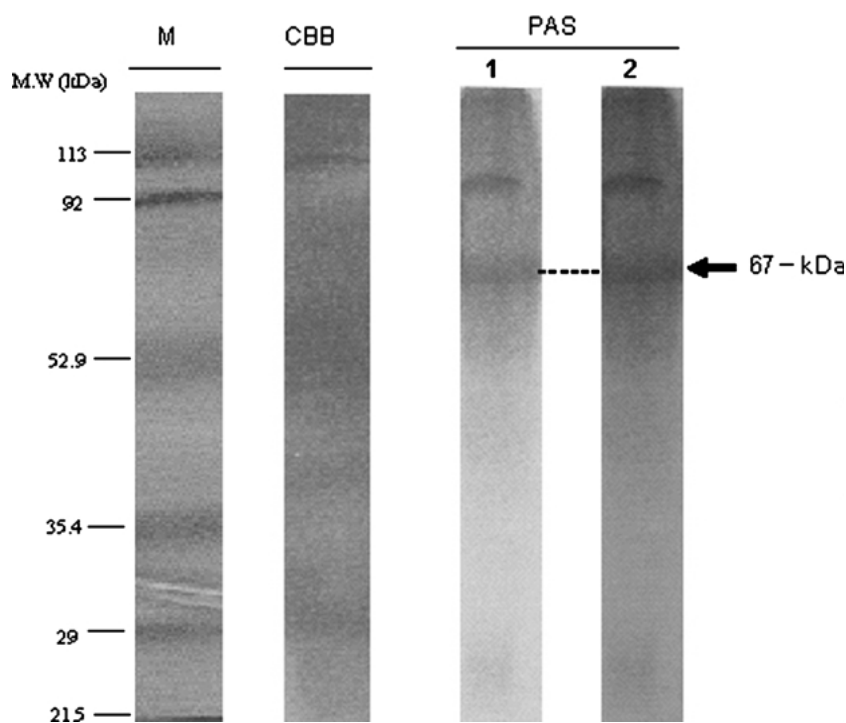


Fig. 2. Identification of glycoproteins by PAS staining. Lanes 1 and 2 are stained for glycoprotein with PAS for insoluble and soluble matrix fractions, respectively. An eluate (derived from 10 g of calcitic sclerites from both samples) was run on 12% polyacrylamide gel and transblotted onto a PVDF membrane. *Arrow* indicates a glycoprotein at approximately 67 kDa. *Broken line* indicates the same protein in the soluble and insoluble matrix fractions. Strong red/purple staining specified the glycoproteins. CBB, CBB staining; M, protein marker; MW, molecular weight.

2.7.1.15); (2) hypothetical protein MJ0304; (3) DNA repair and recombination protein radA; (4) septum formation protein Maf; (5) ribosomal protein L11 methyltransferase (EC 2.1.1.-, L11 Mtase); (6) dehydrin ERD14; (7) chaperone protein high-temperature protein G (heat shock protein htpG).

The calcification process was simulated *in vitro* by the precipitation of CaCO_3 in the presence of proteinaceous

organic matrix from the sclerites of *L. crassum*. The results are shown in Figure 4. Crystals grown in the absence of organic matrix (Fig. 4A) exhibit the characteristic rhombohedral morphology of calcite. Crystal nucleation density was higher when a large volume of matrix was added in a reaction vessel (Fig. 4E). Subsequently, when 25 μg and 1/10 or less of matrix proteins were added to solutions, there was only a slight crystal

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(1) | 48-kDa:      2  EVVF--ELKEE--E-IKDGF-GEF  19
    |           E  VF EL EE E IKD F GE+
    | RBSK_YEAST: 202  ESVFDNELVEEIREKIKDDFLGRY  225

(2) | 48-kDa:      2  EVVFELKKEEIKDGFGE  18
    |           EV  E KEEE K GF E
    | Y304_METJA: 107  EV--EVKEEE-KKGFME  120

(3) | 48-kDa:      1  GEVVFELKKEEIKD  14
    |           GEVVF L EE I+D
    | RADA_SULSO: 308  GEVVFALTEEGIRD  321

(4) | 48-kDa:      2  EVVF-ELKKEEIKDGF  16
    |           EV F EL EEEI D +
    | MAF_BACAN: 122  EVTFWELTEERI-DAY  136

(5) | 48-kDa:      2  EVVFELK--EEEI--K---DGFGEFA  20
    |           E  VF LK EEE+  K  +GF FA
    | PRAMA_THENE: 5  ELVFLKIDEEELLEKLYEEGFFNFA  30

(6) | 48-kDa:      2  EVVFELKKEEIKDGFGE  18
    |           EV  E KEEE K GF E
    | ERD14_ARATH: 107  EV--EVKEEE-KKGFME  120

(7) | 48-kDa:      1  GEVVFELKKEEIKDGF---GEF  19
    |           G  F  KE EIKDGF  G+F
    | HTPG_CLOPE: 98  GSLQFK-KENEIKDGFDIIGQF  118

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Fig. 3. Alignment identified by WU-BLASTP program between N-terminal sequences of the 48 kDa protein and other proteins obtained from the Swissprot database. Sequence was compared with high scoring segment pairs ranked. Swissprot accession numbers: (1) P25332, (2) Q57752, (3) Q55075, (4) Q81LD6, (5) O86951, (6) P42763, (7) Q8XNC2.

growth increase (Fig. 4B-D). Crystal grown in the presence of matrix protein was examined by scanning electron microscopy (SEM) (Fig. 4B-E), indicating the matrix protein of sclerites from *L. crassum* can control crystal growth and a potential template for sclerite formation.

Discussion

The present study has revealed that the calcitic sclerites of *L. crassum* contain water-soluble and -insoluble organic matrices. The soluble matrix may be present as an intracrystalline matrix as suggested in mollusk shells [3]. Accordingly, the insoluble matrix corresponds to the intercrystalline matrix observed after decalcification of thin sections of spicules [1]. Although several investigators have distinguished the amino acid compositions of the soluble and insoluble fractions of mollusk shells, until now no direct evidence has been advanced on the same fractions of calcareous structures of alcyonarians. The soluble fraction in the clam *Mercenaria mercenaria* was found to be approximately 15% of the total organic matrix [22], but in other mollusks this fraction may be up to 64% [23]. In the soluble matrix of *L. virgulata* and the total matrix of alcyonarians studied by Mitterer [9],

the aspartate residues are the most abundant, occurring in excess of 65% of the total amino acids present. Twenty percent of *L. virgulata*'s insoluble matrix is composed of aspartate. However, some similarities can be recognized between these two studies. The aspartate concentration of the insoluble matrix of *L. crassum* is approximately the same but lower in the case of soluble matrix, decreasing the values reported by Mitterer [9]. Mitterer [9] found that glycine and alanine were next in abundance, followed by glutamate, while *L. crassum* showed a different order: alanine and glycine, followed by glutamate. However, alanine occurred in greater and glutamate in smaller quantities than those observed by Mitterer [9]. Therefore, differences seen between the present and previous studies may be attributed to the separation of the soluble and insoluble fractions of the former compared to the total organic matrix of the latter. Differences may also be attributed to individual and/or species variations.

In scleractinian corals, Young [14] found that aspartate, glutamate, and glycine are the most abundant residues, comprising 40 mol%, while those of *L. crassum* demonstrated 56 mol% and 41 mol% in the soluble and insoluble fractions, respectively. These differences between alcyonarian and scleractinian corals may be due to their different modes of calcification [9]; that is, most

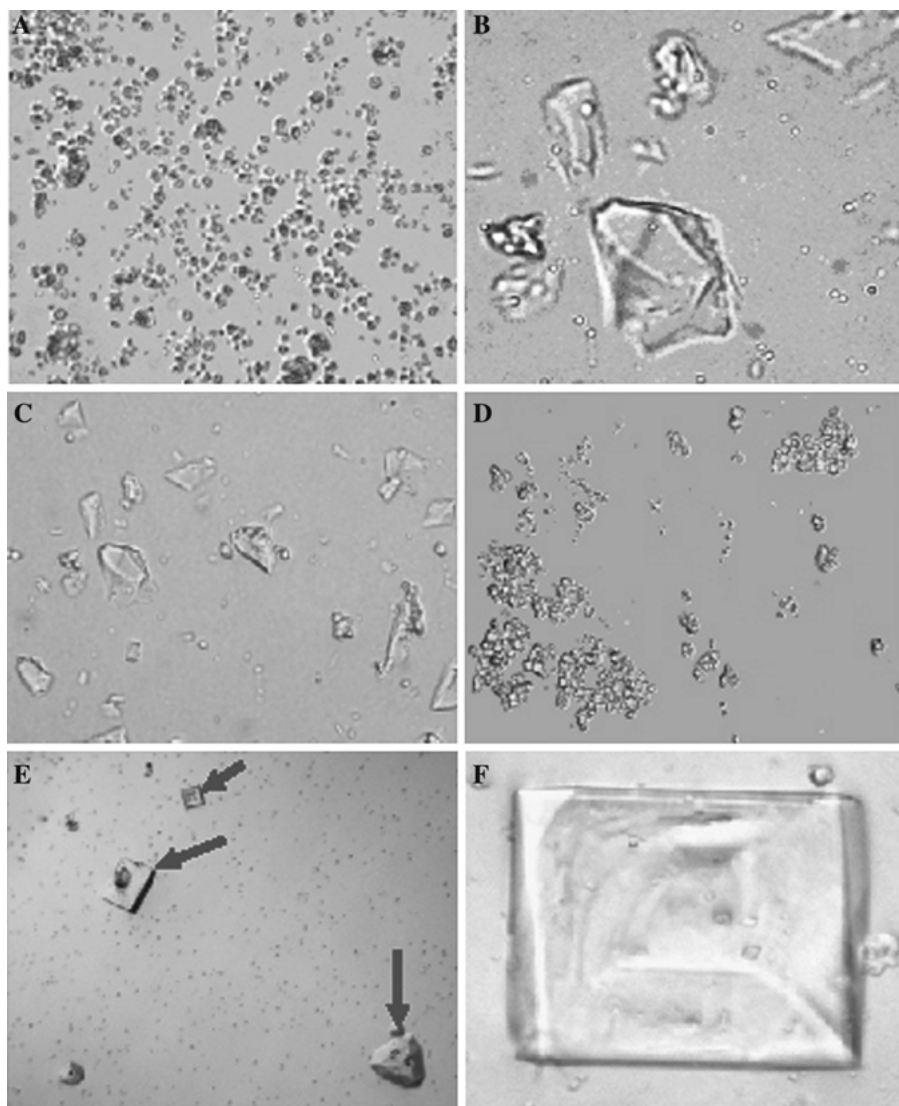


Fig. 4. SEM of crystals grown on the nucleating protein sheet in the presence or absence of polyanionic proteins isolated from calcitic sclerites. (A) Calcite rhombohedrons grown in the absence of polyanionic proteins. (B) Calcite crystals grown in the presence of polyanionic proteins (protein concentration 25 μg). (C) Calcite crystals grown in the presence of polyanionic proteins (protein concentration 2.5 μg). (D) Calcite crystals grown in the presence of polyanionic proteins (protein concentration 1.6 μg). (E) Calcite crystals grown by the same procedure as in B-D but in the presence of large-volume (45 μg) polyanionic proteins were few (arrows), indicating that the proteins can control crystal growth. Calcite was identified according to previous work [33]. (F) SEM of calcite in extended view, which reveals that the resulting new crystal growth was specific to the faces of the calcite rhombohedron. Scale bars: A, B = 25 μm ; C, E, F = 20 μm ; D = 23 μm .

of the process of alcyonarian calcification occurs intracellularly [1], while scleractinian corals calcify extracellularly (see Johnston [24] for review). In all studies of mollusks, the acidic residues (asp, glu) are more abundant in the soluble than the insoluble fractions [25, 26]. This trend is also seen with particular prominence in *L. crassum*. The aromatic amino acid content of the insoluble matrix fraction of mollusks was found to be approximately two and a half to three times as much as that of the soluble fraction [25, 26]. In *L. crassum*, the aromatic amino acid content of the insoluble matrix fraction is approximately two times greater than that of the soluble fraction. Tyrosine is associated with quinine tanned proteins, which may relate to the insolubility of these matrix fractions [25]. The residue of tryptophan was totally absent in both cases, and the reason for this absence is not identified.

The presence of several discrete bands in both the soluble and insoluble matrices of *L. crassum* upon separation by SDS-PAGE indicates a heterogeneous mix-

ture of proteins. Regarding this, heterogeneous matrices have been described in alcyonarian and scleractinian corals [9, 13], mollusk shells [27], and coccoliths [28]. Weiner et al. [27] believe that the heterogeneity of the soluble matrix may be indicative of its capability to perform diverse functions. Both the soluble and insoluble fractions of *L. crassum* show protein bands at approximately 67 and 48 kDa. The soluble fraction yielded additional bands at 102 and 37 kDa. This may indicate a greater degree of heterogeneity of the soluble fraction, or the staining in the insoluble fraction may have been too weak to distinguish. The bands which stained for protein with CBB were always reproducible at the above molecular weights. The bands, however, varied in intensity from gel to gel. This phenomenon may be explained and even expected for a variety of reasons: (1) tissue was stripped from all parts of the alcyonarian colony, which encompassed sclerites of a variety of growth stages; (2) many colonies were used, which imposed age differences and individual variabil-

ity; (3) Degens [16] and Meenakshi et al. [11] have shown in mollusks that the amino acid composition of shells varies within a species according to its environmental conditions. Johnston [24] has found that in scleractinian corals there is a natural degradation of matrix amino acids, a process which may also occur in alcyonarians.

CBB and PAS stained both matrix fractions at the position of 67 kDa, indicating the presence of glycoproteins at this molecular weight. Weiner et al. [17] reported that the organic matrix of *Leptogorgia* is a glycoprotein of approximately 99-100 kDa. Crenshaw [22] found that the soluble matrices of *M. mercenaria* were glycoprotein with a molecular weight of 160 kDa. Tompa et al. [29] described the insoluble matrix of snail eggshell as a sulfated glycoprotein of 53 kDa. The glycoproteins of mollusks [22, 23] and the polysaccharides of coccoliths [28] have been shown to be capable of specifically binding calcium ions. Crenshaw [22] has proposed that calcium binding in the soluble matrix of *Mercenaria* occurs by chelating by ester sulfate groups from two adjacent polysaccharide chains. Although a recent finding of Rahman and Isa [13] identified the calcium ion in the soluble matrices of sclerites in alcyonarians, more detailed work needs to be conducted for both fractions. However, these initial gel electrophoretic results indicate that such a scheme of calcium binding is potentially possible for both fractions in *L. crassum*. Further investigation may determine which factors, i.e., acidic amino acids or glycoproteins, are responsible for calcium binding in alcyonarians.

Our previous findings [13] and the present investigation have revealed that the sclerites of *L. crassum* contain protein-rich organic matrices. These matrices are capable of making protein bands and are subsequently able to positively distinguish amino acid sequences. The amino acid sequence observed in the nacreous layers of oyster pearls (*Pinctada fucata*) was that of a functional domain Gly-Xaa-Asn (Xaa = Asp, Asn, or Glu) type [30], which supports the present results. We identified the alignment of the 48 kDa protein with seven proteins of other phyla from the Swissprot database in which significant identities were found. The RBSK_YEAST, Y304_METJA, RADA_SULSO, MAF_BACAN, PRAMA_THENE, ERD14_ARATH, and HTPG_CLOPE proteins are, respectively, 58% (14/24), 76% (10/13), 71% (10/14), 62% (10/16), 50% (13/26), 64% (11/17), and 54% (12/22) identical to the 48 kDa protein, where the numbers in parentheses indicate the number of identities and the length of the aligned protein. The best match (76%) was with hypothetical protein MJ0304 (Y304_METJA). Matches to specific proteins were characterized by 50% or more identity over much of the N-terminal fragment (Fig. 3). The observation prompted us to detect or demonstrate homology between our new sequences and existing families of sequences, to help predict the secondary and tertiary structures of our novel sequences,

and to suggest oligonucleotide primers for polymerase chain reaction and was an essential prelude to molecular evolutionary analysis. Also, close homology of this protein has been found with that of an organism in another phylum, which is an important feature for this phylum. In this report, it has been established that similarities in the alcyonarian endoskeletal and other phylum's proteins are required, if borne out by further sequence analysis. It is our hope that further sequence studies will provide both the total sequence of some of the proteins and partial sequences from a larger number of proteins and thereby contribute to a better understanding of structural proteins in alcyonarians.

The proteinaceous organic matrix of sclerites plays a major role in the biomineralization process [12]. Weiner et al. [17] propose that the insoluble matrix acts primarily as a nonmineralizing structural framework and/or crystal growth inhibitor on which the soluble fraction is aligned. Samata et al. [31], on the other hand, found that although aspartic acid and glutamic acid were abundant in the soluble matrices of mollusk shells, these amino acids were not predominant in its calcium binding fractions. In addition, previous studies on molluscan shells indicated that acidic amino acid residues may actually inhibit crystal nucleation [32]. Our present investigation has revealed that both fractions of alcyonarians, which contain a high volume of acidic proteins, can regulate the crystal growth and morphology or induction of crystal nucleation. Finally, the results support the concept that the proteinaceous organic matrix acts as a template which initiates and controls crystal growth.

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