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Identification of a silicatein(-related) protease in the giant spicules of the deep-sea hexactinellid *Monorhaphis chuni*

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SUMMARY

Silicateins, members of the cathepsin L family, are enzymes that have been shown to be involved in the biosynthesis/condensation of biosilica in spicules from Demospongiae (phylum Porifera), e.g. *Tethya aurantium* and *Suberites domuncula*. The class Hexactinellida also forms spicules from this inorganic material. This class of sponges includes species that form the largest biogenic silica structures on earth. The giant basal spicules from the hexactinellids *Monorhaphis chuni* and *Monorhaphis intermedia* can reach lengths of up to 3 m and diameters of 10 mm. The giant spicules as well as the tauactines consist of a biosilica shell that surrounds the axial canal, which harbours the axial filament, in regular concentric, lamellar layers, suggesting an appositional growth of the spicules. The lamellae contain 27 kDa proteins, which undergo post-translational modification (phosphorylation), while total spicule extracts contain additional 70 kDa proteins. The 27 kDa proteins cross-reacted with anti-silicatein antibodies. The extracts of spicules from the hexactinellid *Monorhaphis* displayed proteolytic activity like the silicateins from the demosponge *S. domuncula*. Since the proteolytic activity in spicule extracts from both classes of sponge could be sensitively inhibited by E-64 (a specific cysteine proteinase inhibitor), we used a labelled E-64 sample as a probe to identify the protein that bound to this inhibitor on a blot. The experiments revealed that the labelled E-64 selectively recognized the 27 kDa protein. Our data strongly suggest that silicatein(-related) molecules are also present in Hexactinellida. These new results are considered to also be of impact for applied biotechnological studies.

Key words: sponges, *Monorhaphis chuni*, spicules, biosilica, silicatein-related protein.

INTRODUCTION

The phylogenetically oldest metazoans are the sponges (phylum Porifera), which evolved during the Neoproterozoic, Neoproterozoic–Cambrian period (Xiao et al., 2005). Porifera are grouped into three classes, the Hexactinellida and the Demospongiae being both composed of a siliceous skeleton, as well as the class of Calcarea whose skeleton is made of calcium carbonate (Bergquist, 1978). Fossil records (Brasier et al., 1997; Steiner et al., 1993) and 'molecular clock' analyses (Schäcke et al., 1994; Kruse et al., 1998) indicate that the Hexactinellida evolved before to the Demospongiae, between 665 and 650 million years ago. Both sponge classes, which share a common ancestor, appeared prior to one major 'snowball earth event', the Varanger–Marinoan ice age [605 to 585 million years ago (Hoffman et al., 1998)] when the earth was covered by an almost continuous ice layer. This caused extinction of most metazoans except the Porifera. Sponges are distinguished from the other Pre-Cambrian metazoans that are very likely to have existed, the conspicuously ornamented acritarchs found in the Ediacara, 630 to 530 million years ago (see Butterfield, 2007). During the Varanger–Marinoan ice age the oxygen tension reached almost present atmospheric levels (see Fenchel, 2002). Simultaneously a noteworthy geochemical process occurred termed silicate weathering–carbonate precipitation cycle; the dissolution of surface rocks composed of insoluble silicates ($CaSiO_3$) resulting in the formation of soluble calcium carbonate ($CaCO_3$) and soluble silica (SiO_2), under consumption of atmospheric CO_2 (Walker, 2003).

So far data indicate that demosponges have the unique ability to synthesize their siliceous skeleton enzymatically (Shimizu et al., 1998; Cha et al., 1999), in contrast to other organisms that deposit bio-silica in a template-controlled manner (reviewed in Perry, 2003). The responsible enzyme, silicatein, was first described by the group of Morse in the marine demosponge *Tethya aurantium* (Cha et al., 1999) and subsequently also identified in other demosponges, most prominently in *Suberites domuncula* (Krasko et al., 2000). The genes of two isoforms of silicateins, silicatein-α and silicatein-β (Cha et al., 1999), have been identified in marine demosponges, and a third type, silicatein-γ, has been identified but not yet at the gene level (Shimizu et al., 1998). The silicateins undergo post-translational modification, primarily phosphorylation (Müller et al., 2005). The polypeptide sequences of the silicateins share high sequence similarity with cathepsin L (Cha et al., 1999; Krasko et al., 2000); the active centre of silicatein differs from that of cathepsin L by only one amino acid (catalytic triad is Ser-His-Asn in silicatein and Cys-His-Asn in cathepsins). Cathepsins belong to the cysteine proteinases which can be effectively

inhibited by E-64 [L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane] (Barrett et al., 1982) (reviewed in Barrett et al., 2002); E-64 does not affect cysteine residues in other enzymes, including the serine proteinases. The natural cathepsin inhibitor cystatin reduces the activity of cathepsins potently (reviewed in Brage et al., 2005; Laitala-Leinonen et al., 2005).

The first investigations focusing on the process of silica formation in Hexactinellida have only very recently been published (Ehrlich et al., 2006; Müller et al., 2007b; Wang et al., 2007). Typically, this class of sponges comprises hexactine spicules with three axes intersecting at right angels; loss of one or more rays often occurs and leads to pentactine, tetractine, triactine, diactine or monactine forms (Reiswig, 2006). Two types of spicule exist in Hexactinellida, megascleres and microscleres, which are grouped according to their form, size and function. According to the different types of microsclere, two main lineages, Amphidiscophora (amphidiscs) and Hexasterophora (hexasters) have been distinguished (see Reiswig, 2006). While all Amphidiscophora have a skeleton with distinct, non-fused spicules, the spicules of the Hexasterophora often fuse. The composition and morphology of the Hexactinellida have been described very thoroughly, starting as early as 1832 (Gray, 1832) and are still studied today (Aizenberg et al., 2005; Weaver et al., 2007). In spite of the highly remarkable diversity of the hexactinellid spicules, they represent only one level of hierarchy in their skeletal system (Aizenberg et al., 2005).

One hexactinellid family, the Monorhaphididae, comprises the genus *Monorhaphis*, which includes the species *Monorhaphis chuni* (Schulze, 1904), *Monorhaphis dives* (Schulze, 1904) and *Monorhaphis intermedia* (Li, 1987), which form the longest bio-inorganic siliceous structures on earth (Tabachnick, 2002). Their cylindrical bodies are stabilized by microscleres and megascleres. Most interestingly, the body develops around a giant basal spicule, which can grow up to a length of 3 m with a diameter of 10 mm (Tabachnick, 2002). The spicules grow in a lamellar way, through the concentric deposition/formation of silica layers; in the centre of the spicules an organic axial filament is harboured in a rectangular axial canal (reviewed in Schulze, 1904; Schulze, 1925; Reiswig, 1971; Sandford, 2003). The megascleres of *M. chuni* and *M. intermedia* consist of up to 400 lamellae (Levi et al., 1989). It should be highlighted that even as early as 1860 (Schultze, 1860) and 1878 (Chimmo, 1878) the lamellar construction of the hexactinellid spicules had already been clearly demonstrated.

Here we examined the biochemical composition of the megascleres (giant basal spicules; size, 1.2 m) as well as of the tauactines (size, 1 mm) from *M. chuni* and *M. intermedia*, which are very closely related species (Li, 1987; Tabachnick and Lévi, 2000; Tabachnick, 2002). The giant basal spicules and the tauactins display the same lamellar organization. While in one specimen only one giant basal spicule exists that supports and fixes the oval, spindle-like body to the substratum, many tauactins are found in the choanosomal skeleton (Schulze, 1904; Li, 1987; Tabachnik and Lévi, 2000). We focused especially on the potential existence of silicatein, or silicatein-like molecules in the two types of spicule. We demonstrate for the first time that the protein(s) present in the spicules of *M. chuni* display proteolytic activity, like the silicateins from *S. domuncula;* this indicates/suggests that the spicules in Hexactinellida are also synthesized enzymatically by silicatein-like molecule(s). To further characterize the proteinase, we used the naturally occurring inhibitor E-64 and the sponge inhibitor cystatin. The gene encoding cystatin was obtained from *S. domuncula* and prepared in a recombinant manner.

MATERIALS AND METHODS

Materials

Casein and Coomassie Brilliant Blue were obtained from Serva (Heidelberg, Germany); alkaline phosphatase (AP)-labelled anti-rabbit IgG, isopropyl β-D-thiogalactopyranoside (IPTG), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC no. 22980) and EZ-link amine-PEO-biotin labelling reagent (no. 21346) were from Pierce (Rockford, IL, USA); E-64 was from Sigma-Aldrich (Taufkirchen, Germany); Z-Phe-Arg-AMC was from Bachem (King of Prussia, PA, USA); and cathepsin L (specific activity, 6354 mU mg^{-1} protein) was from Calbiochem (San Diego, CA, USA).

Sponges

Spicules from the hexactinellids *Monorhaphis chuni* [(Schulze, 1904) Porifera: Hexactinellida: Amphidiscosida: Monorhaphididae] and *Monorhaphis intermedia* [(Li, 1987) Porifera: Hexactinellida: Amphidiscosida: Monorhaphididae] were used. Recently, it has been proposed that *M. chuni* and *M. intermedia* are one species (Tabachnick and Lévi, 2000; Tabachnick, 2002). The *M. chuni* specimens used were provided from the collection of the Museum für Naturkunde Berlin (Germany). They were collected by deep-sea dredging in 1899 at a depth of 1700 m during the 'Valdivia' expedition in the Somali basin (ZMB Por 3708, 4253, 12700). *M. intermedia* was collected during a Chinese expedition in 1981 to the West-Pacific Okinawa Trough at a depth of 900 m (Jinhe Li). Both giant basal spicules and tauactins were used from them.

Specimens of the marine demosponge *Suberites domuncula* (Olivi 1792; Porifera: Demospongiae: Hadromerida: Suberitidae) were collected in the Northern Adriatic near Rovinj (Croatia), and then kept in aquaria in Mainz (Germany).

Spicules and spicule extracts

Spicules were soaked in 2% (w/v) sodium dodecyl sulphate solution overnight to remove cell and tissue remains. The spicules were treated with 50 ml of 2 mol l^{-1} HF (hydrofluoric acid)/8 mol l^{-1} NH_4F (pH 5) for only 3 h (Shimizu et al., 1998). Then the suspension was immediately dialysed (3 times) against 5 l of 50 mmol l^{-1} Tris-HCl buffer (pH 9.0; 100 mmol l^{-1} NaCl, 10 mmol l^{-1} EDTA) at 4°C for 4 h each. Subsequently, the extract was concentrated with Microcon centrifugal filter devices (cutoff, 3 kDa, 3000 MW cutoff; Millipore, Schwalbach, Germany) and finally frozen at –20°C until analysis.

For the visualization of nanoparticles that make up the giant basal spicules, the cut spicules were etched with HF vapour. For the analysis of the polypeptides within siliceous structures, single lamellae were mechanically separated from the other layers of the giant basal spicules and extracted separately.

Spicules from *S. domuncula* were obtained and the axial filaments isolated by the 2 mol l^{-1} HF/8 mol l^{-1} NH_4F procedure as described previously (Schröder et al., 2006). The filaments were treated with phosphate-buffered saline (PBS; containing 1% Triton X-100) and used for the determination of the proteinase activity.

SDS-PAGE and western blot analysis

Samples from spicules containing 1–3 μg of protein were dissolved in loading buffer (Roti-Load; Roth, Karlsruhe, Germany), boiled for 5 min and then subjected to 10% polyacrylamide gel electrophoresis, containing 0.1% sodium dodecyl sulphate (SDS-PAGE). After protein separation the gels were washed in 10%

methanol (supplemented with 7% acetic acid) for 30 min and then stained in Coomassie Brilliant Blue as described previously (Müller et al., 2005).

For western blot analysis, the polypeptides were transferred from the polyacrylamide gel to a nitrocellulose membrane (pore size 0.45 μm; no. T831.1, Millipore, Schwalbach, Germany) using the Trans-Blot SD system (Bio-Rad). The membrane was rinsed in TBS-T (20 mmol l^{-1} Tris-HCl pH 7.6, 137 mmol l^{-1} NaCl, 0.1% Tween-20) and incubated for 1 h with rabbit polyclonal anti-silicatein antibody (PoAb-aSilic, no. N365) that had been raised against the silicatein from *S. domuncula*, as described earlier (Müller et al., 2005). The dilution of the antiserum was 1:1000. The membranes were washed three times in TBS-T and then incubated for 1 h with biotinylated goat anti-rabbit IgG secondary antibody (no. 111-035-144, Jackson ImmunoResearch, Newmarket, Suffolk, UK). VECTASTAIN ABC Kit (PKG-100; Linaris, Wertheim, Germany) was used for signal enhancement. For visualization, the peroxidase substrate kit TMB (no. SK-4400, Linaris Biologische Produkte GmbH, Wertheim, Germany) was used. In a control experiment 100 μl of the PoAb-aSilic was adsorbed with 0.1 mg of recombinant silicatein (Müller et al., 2005) for 30 min at 4°C prior to use.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (IEF) was carried out as described previously (Coligan et al., 1998) using a Protein-IEF chamber (Bio-Rad) and immobilized pH gradient (IPG) strips (ReadyStrip IPG Strip, pH 3–10, Bio-Rad). Samples containing 30 μg protein were mixed with rehydration buffer [8 mol l^{-1} urea, 0.4% ampholytes, 60 mmol l^{-1} 1,4-dithio-DL-threitol (DTT), 0.002% Bromophenol Blue] and then loaded onto the strips (Coligan et al., 1998). The molecular mass markers All Blue Standards (no. 161-0373, Bio-Rad) and isoelectric point (pI) standards 2-D SDS-PAGE Standards (no. 161-0320, Bio-Rad) were used. Staining of proteins was performed with Coomassie Brilliant Blue.

Determination of the active centre with biotinylated E-64

Aliquots of 10 μl (containing approximately 20 μg of protein) of a soluble extract from *Monorhaphis* spicules were mixed with 10 μl of acetate buffer (pH 5.5; 50 mmol l^{-1} sodium acetate, 100 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA) and incubated for 10 min at room temperature for activation. Then 2 μl of biotinylated E-64 (final concentration in the assay: 50 μmol l^{-1}) were added to this sample and incubated at 22°C for 1 h. Subsequently, 5 μl aliquots were added to 4 μl of 6× Laemmli sample buffer (Laemmli, 1970), containing 5% β-mercaptoethanol, and heated at 95°C for 5 min prior to loading onto the gel. The samples were analysed by SDS-PAGE (10% gel) (Laemmli, 1970) and stained with Coomassie Brilliant Blue. In parallel, the size-separated proteins were transferred onto nitrocellulose membrane. The biotinylated E-64 was obtained from unlabelled E-64 by coupling the inhibitor via amine-PEO_2-Biotin (Pierce, Rockford, IL, USA) and using the cross-linker 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), following the manufacturer's instructions. For the identification of potential E-64 binding sites on *Monorhaphis* proteins, 10 μl aliquots of a soluble spicule fraction were mixed with 2 μl of 50 mmol l^{-1} sodium acetate buffer (pH 5.5; 100 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA) and incubated for 10 min at room temperature for activation. Then 2 μl (50 μmol l^{-1} final concentration) of biotinylated E-64 were added to the activated enzyme and the mixture was incubated at 22°C for 1 h. The samples were mixed with 4 μl of 6× Laemmli sample buffer, containing 5% β-mercaptoethanol, and heated for 5 min at 95°C. Samples were analysed by SDS-PAGE (10% gels) as above. After size separation, blotting to nitrocellulose membranes (Bio-Rad, München, Germany) was performed. Incubation with biotin/avidin Vectastain Elite ABC [Vector Laboratories, Burlingame, CA, USA (Hsu et al., 1981)] was carried out prior to the detection of the signals by the chemiluminescence procedure using Immobilon Western horseradish peroxidase substrate (Luminol reagent, Millipore).

Electron microscopy

Scanning electron microscopic (SEM) analysis of spicules was performed with a Zeiss DSM 962 digital scanning microscope (Zeiss, Aalen, Germany). The samples were mounted onto aluminium stubs (SEM-Stubs G031Z; Plano, Wetzlar, Germany) that had been covered with adhesive carbon (carbon adhesive Leit-Tabs G3347, Plano, Wetzlar, Germany). Then the samples were sputtered with a 20 nm thin layer of gold in argon plasma (Bal Tec Med 020 coating system; Bal Tec, Balzers, Liechtenstein). The surfaces of the cross-sections were polished with emery paper (silicon carbide; Matador, Hoppenstedt, Darmstadt, Germany) and the quality of the surface was inspected under a stereomicroscope with an enlargement of about ×30. Backscattered analysis was performed with this microscope using 15 keV beam voltage and 50 μA emission current at a working distance of 6 mm (Holmes et al., 1987). Partial etching of the surfaces of the spicules was performed with 1% HF for 5 min.

Cloning of cystatin cDNA from *S. domuncula*

For the isolation of the cDNA encoding cystatin, a cDNA library of *Suberites domuncula* was used (Kruse et al., 1997). From this a set of 30 000 expressed sequence tag sequences has been compiled. The complete cDNA coding for cystatin (CYTA_SUBDO), *SUBDOCYTA*, was obtained by PCR and deposited on the EMBL/GenBank database (accession no. AM411124). Fragments were cloned into the Topo TAII vector in *Escherichia coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA). Sequencing was performed with primers directed to the SP6 and the T7 promoters. The sequence was completed with insert-specific primers in combination with T7 and SP6 primers. The final sequence was confirmed by an additional PCR using primers directed against the non-translated region of the cDNA, followed by sequencing. The clone encoding the *S. domuncula* cystatin was 510 nucleotides (nt) long [excluding the poly(A) tail].

Recombinant sponge cystatin

The sponge *SUBDOCYTA* sequence was expressed in *E. coli* cells, strain BL21. The complete open reading frame (ORF; nt 46–330), was isolated by PCR using one forward primer (5′-GGATCCTC-TGCGACAGAACAAGCACTAGTGGG-3′; the *Bam*HI restriction site is underlined) and one reverse primer (5′-CTCGAGACTGG-TTAGGTTAGGTAGTAGAAAGACACG-3′; the *Xho*I restriction site is underlined). The 285 bp long section was cloned into the expression vector pET 41a, which contained at the 5′-terminus the GST (glutathione S-transferase) tag and the polyhistidine region. The insert was expressed in *E. coli* BL21 cells overnight at 30°C in the presence of 0.1 mmol l^{-1} IPTG. The fusion protein was extracted and purified with the His-tag purification kit (Novagen, Madison, WI, USA). The purity of the material was checked on 10% polyacrylamide gels containing 0.1% SDS (PAGE) according to Laemmli (Laemmli, 1970).

Sequence analyses

The sequences were analysed with the computer programs BLAST (2005; http://www.ncbi.nlm.nih.gov/blast/blast.cgi) and FASTA (2005; http://www.ebi.ac.uk/fasta33/). Multiple alignments were performed with CLUSTAL W version 1.6 (Thompson et al., 1994). Phylogenetic trees were constructed on the base of amino acid sequence alignments by neighbour joining, as implemented in the Neighbor program from the PHYLIP package (Felsenstein, 1993). The distance matrices were calculated using the Dayhoff PAM matrix model as described previously (Dayhoff et al., 1978). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein, 1993). The graphic presentations were prepared with GeneDoc (Nicholas and Nicholas, 1997).

Analysis of proteolytic activity: zymogram analysis

Protein samples (axial filaments) were obtained from *S. domuncula*, dissolved in PBS/Triton X-100 (see above), and loaded onto a zymogram gel, containing 0.1% casein (heat denatured) as previously described (Jaffe and Dwyer, 2003). The samples were separated by SDS-PAGE (10% gels). Samples (15 μl) were loaded onto each gel, corresponding approximately to a protein extract from 200 μg of spicules. After protein separation, the gels were incubated in a 50 mmol l^{-1} Mops buffer (pH 6.8; 5 mmol l^{-1} $CaCl_2$, 0.1 mmol l^{-1} $ZnCl_2$, 100 mmol l^{-1} NaCl, 0.5 mmol l^{-1} DTT) for 1 h at room temperature. After refreshing this buffer, proteinase activity was allowed to develop overnight at 37°C; then the gels were stained with Coomassie Brilliant Blue in order to visualize the proteinase activity as clear bands on a blue background. Where indicated, the *S. domuncula* spicule extracts were preincubated (30 min; 20°C) with 1 μmol l^{-1} E-64 or 5 μg ml^{-1} of recombinant *S. domuncula* cystatin.

Analysis of proteolytic activity: enzyme activity test

Extracts from spicules of both *M. chuni* and *S. domuncula* were obtained from purified spicules after dissolution of the bio-silica shell with HF/NH_4F as described above. The enzymatic reaction (0.2 ml volume) was performed as described elsewhere (Quian et al., 1989; Mort, 2002) in 96-well plates (Nunc 96 Microwell™ Plates, Nunc, Wiesbaden, Germany) at room temperature. Cathepsin L was used as a positive control at a final concentration of 10 nmol l^{-1}. The assay contained 10 μmol l^{-1} Z-Phe-Arg-AMC as substrate. Incubation was performed for 60 min at room temperature. Then the spicule samples were pre-incubated either with 1 μmol l^{-1} E-64 or with 5 μg ml^{-1} of recombinant *S. domuncula* cystatin. A standard curve was established with 7-amino-4-methyl-coumarin (AMC) under otherwise identical incubation conditions. The fluorescence of the free AMC released was determined using excitation at 355 nm and emission at 460 nm in an F-2000 Hitachi fluorescence spectrophotometer as described previously (Dvorak et al., 2005). The activity was calculated and is given in nmol AMC released mg^{-1} protein min^{-1} (Dvorak et al., 2005). Five parallel experiments were performed; the means and the standard deviations were calculated (Sachs, 1984).

Analytical method

The Bradford method (Roti-Quant solution; Roth) was used for protein quantification (Compton and Jones, 1985).

RESULTS

Lamellar organization of the spicules (giant basal spicules and tauactins) from *Monorhaphis*

The diameters of the giant basal spicules used here were 1–6 mm, and those of the tauactins were a maximum of 25 μm; Fig. 1. Giant basal spicules and tauactins of *M. chuni* and *M. intermedia* were covered by a sheath/layer of collagen; a ray of a tauactin is shown in Fig. 1A. The silica shells of the spicules were constructed of up to 20 lamellae for the tauactins (Fig. 1A,C,D) and approximately 400 lamellae for the giant basal spicules (Fig. 1B). The layers of the tauactins were around 1–2 μm thick (Fig. 1C,D), while those in different giant basal spicules were between 2 and 10 μm (Fig. 1B). The thickness of the lamellae along one given spicule was almost constant (ranging between 1 and 4 μm). The silica layers were separated from each other by 20–50 nm wide gaps (Fig. 1E,F), which were presumably initially filled by an organic material. The axial canal was rectangular and filled with an axial filament of the same shape (Fig. 1G–I). The spicule samples, analysed here, showed that the diameter of the axial canals of the giant basal spicules was less than 1 μm, whilst that of the canals of the tauactins reached more than 3 μm (Fig. 1G,H). Cross-sections of the giant basal spicules revealed that the axial canal was surrounded by silica layers of very dense and homogeneous silica, forming the cylinder (Fig. 1B). The silica material, building the layers of the tauactins, appeared to be of the same density (Fig. 1D). At the tips of the spicules the axial canal with the axial filament remained open and allowed an inspection of the axial filaments (Fig. 1G). If the silica material was etched with HF, the nanoparticles composing the silica shell were exposed (Fig. 1F).

Protein pattern in spicules from *M. chuni*

The surfaces of the giant basal spicules were first mechanically and then chemically cleaned. Later they were either directly processed or the lamellae were prepared and then extracted for further analysis. PAGE analysis of an extract from a total giant basal spicule showed that under the conditions used two sets of proteins exist; (i) high molecular weight triplets at around 70 kDa, and (ii) a set of 27 and 30 kDa (Fig. 2A lane b). If only the lamellae were extracted, only the 27 kDa protein was detected (Fig. 2A lane a).

A two-dimensional gel electrophoretic separation of the total extract revealed a more complex pattern (Fig. 2C). The higher molecular mass polypeptides (70 kDa) split into an acidic and a more basic set of proteins. The more acidic proteins had isoelectric points (pI) between 4.7 and 4.2, while the pI values in the more basic range varied between 6.8 and 7.3 (Fig. 2C). Very interestingly, the 27 and 30 kDa molecules also split into different spots, reflecting proteins with pI values of five isoforms, which are characterized by the following pI values: 6.6, 6.2, 6.0, 5.8 and 5.6 (Fig. 2C). In addition, this analysis showed that the isoform pattern of the 27 kDa polypeptide(s) was highly similar to that of the 30 kDa series, which is more faint. A two-dimensional gel electrophoretic separation of an extract from lamellae was performed in parallel, which revealed a similar isoform pattern to the 27 kDa molecule(s) from the total extract (not shown).

Western blot analysis was performed with an extract from the lamellae, using polyclonal antibodies raised against *S. domuncula* silicatein. In this experiment the 27 kDa polypeptide strongly reacted with these antibodies (Fig. 2B lane a). As a control, the anti-silicatein antibodies were preadsorbed with recombinant silicatein; this sample did not react with any protein species on the blot (Fig. 2B lane b).

Sponge cysteine proteinase inhibitor: cystatin

Natural peptides, belonging to the cystatin superfamily, are potent inhibitors of papain-like cysteine proteinases, e.g. the cystatins and the stefins [see http://merops.sanger.ac.uk (Rawlings et al., 2004)]. These molecules are subdivided into (i) type 1 cystatins,

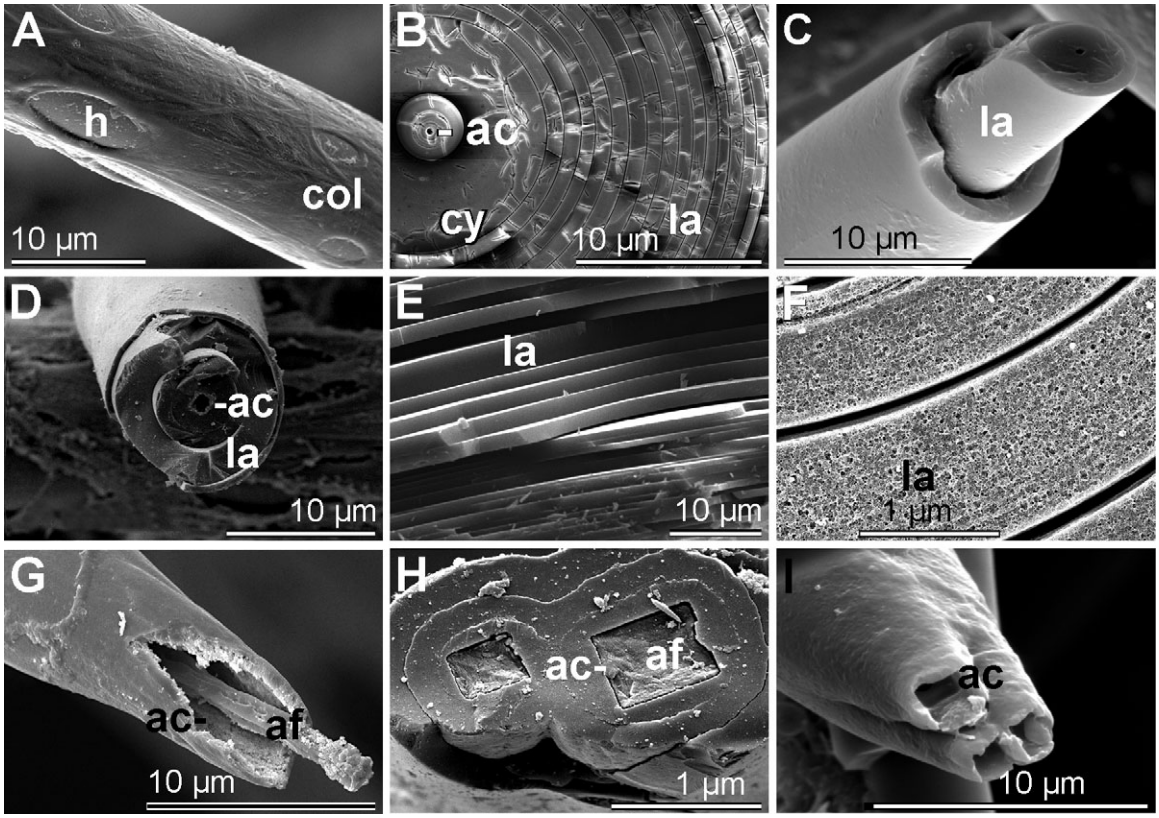

Fig. 1. Megascleres of *M. chuni* (A–D,G–I) and *M. intermedia* (E,F); scanning electron microscopic analysis. (A) The tauactins are covered by a collagen (col) sheath, which is interspersed with 5 µm holes (h). (B) Cross-section through a giant basal spicule showing the outer lamellar zone (la) and the central axial cylinder (cy), which surrounds the axial canal (ac). (C,D) The tauactins are composed of a maximum of 20 lamellae. (E) A stack of lamellae of the outer zone of a giant basal spicule. (F) Cross-section through a giant basal spicule; the surface has been etched with HF, disclosing the composition of the lamellae by silica nanoparticles. (G) The tip of a tauactin, allowing inspection of the axial canal with its axial filament (af). (H) Cut-through section of a tauactin at the position where two rays/spines originate, exposing two axial canals with their axial filaments. (I) Tip of a tauactin, harbouring three axial canals.

cystatin/stefins A and B; these are polypeptides of ~98 amino acid residues that possess neither disulphide bonds nor carbohydrate side chains, and are located mainly intracellularly; (ii) type 2 cystatins C, D, E/M, F, S, SN, and SA; these are characterized by conserved disulphide bridges; and (iii) type 3 cystatins, the kininogens (Kopitar-Jerala, 2006). For the analysis of the proteinase specificity in the sponge spicules, type 1 sponge cystatin was cloned and the recombinant protein prepared.

The sequence for type 1 cystatin, which was termed *SUBDOCYTA*, is 510 nt long and comprises one ORF from nt 43–45 to nt 431–433(stop). The 96 amino acid-long deduced protein (termed CYTA_SUBDO) has a size of 10 828 Da and a pI of 6.4. The sponge cystatin shares highest sequence similarity with the human sequences cystatin A and cystatin B, with an 'expect value' (Coligan et al., 2000) of $3e^{-04}$ [cystatin B (Joensuu et al., 2007); cystatin A (Werle et al., 2006)]. The sponge cystatin

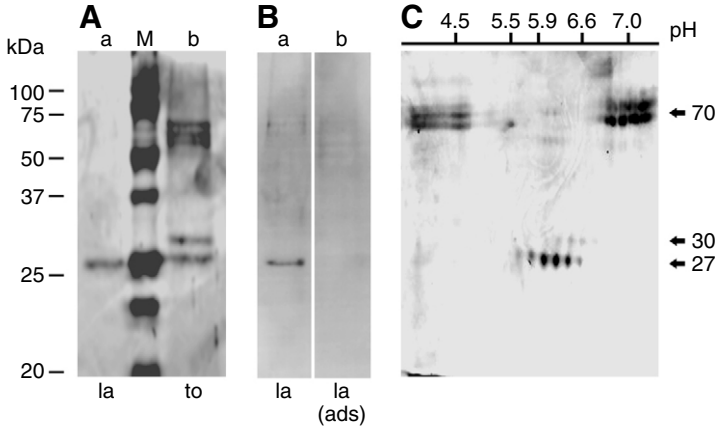

Fig. 2. Analysis of proteins in giant basal spicules from *M. chuni*. (A) Protein extracts were prepared from the outer lamellar region of the spicules (la; lane a) or from total spicule (to; lane b) and size separated by 10% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue. Size markers are given (M). (B) Corresponding western blot from the separation of a protein extract obtained from lamellae; the blot was reacted with polyclonal antibodies raised against silicatein (PoAb-aSILIC) and then with labelled secondary antibodies (lane a). In a separate series, the blot was first incubated with PoAb-aSILIC that had been adsorbed with recombinant silicatein (ads), and then incubated with the labelled secondary antibodies (lane b). (C) Analysis of a total spicule extract by two-dimensional gel electrophoresis (first isoelectric focusing and then size separation). The arrows mark the positions of the two sets of proteins in the total spicule extract, the 27/30 kDa molecules and the 70 kDa polypeptides. The gel was stained with Coomassie Brilliant Blue. Further details are given under Materials and methods.

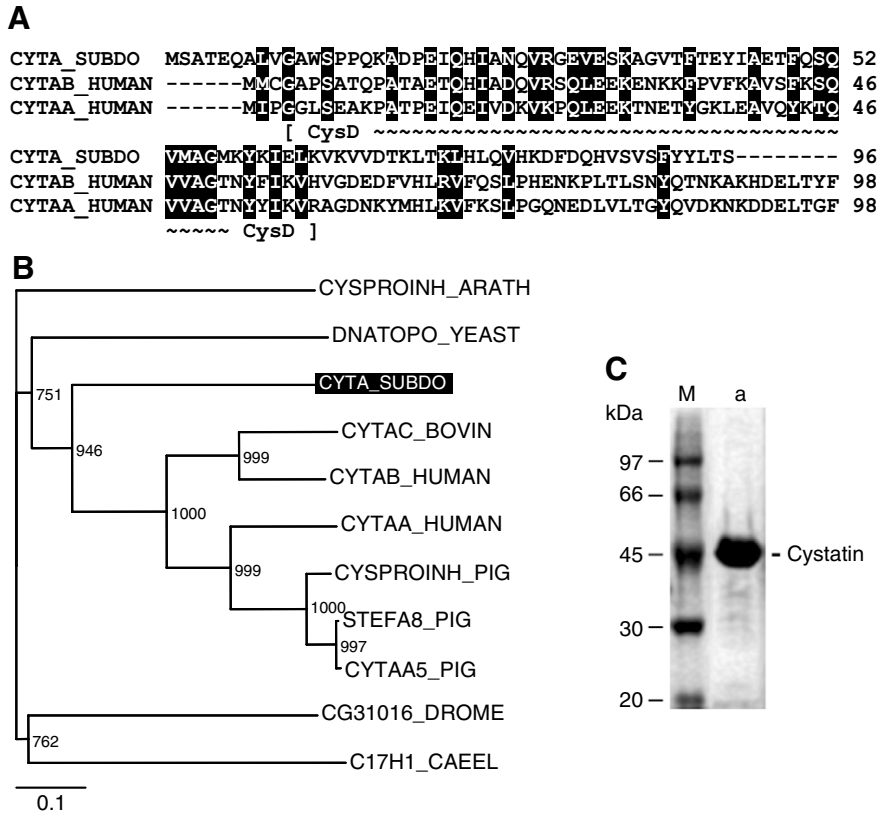

Fig. 3. Sponge cystatin, a cysteine proteinase inhibitor. (A) Alignment of the sponge cystatin (CYTA_SUBDO) with the corresponding cystatins from humans, cystatin A (CYTAA_HUMAN; accession number NP_005204) and cystatin B (CYTAB_ HUMAN; NP_000091). Amino acids that are similar among all three sequences are in white type. The characteristic cystatin domain spans amino acids 11 to 66 (CysD). (B) A phylogenetic tree constructed after alignment of the above-mentioned three sequences as well as the pig leukocyte cysteine proteinase inhibitor 1 (CYSPROINH_PIG; P35479), pig stefin A8 (STEFA8; NP_999025), pig stefin A5 (CYTAA5_PIG; Q28986), cattle proteinase inhibitor stefin-C from *Bos taurus* (CYTAC_BOVIN; P35478), the insect putative protein CG31016 from *Drosophila melanogaster* (CG31016_DROME; NP_733393), the putative protein from *Caenorhabditis elegans* (C17H1_CAEEL; NP_493295), the yeast enzyme DNA topoisomerase III from *Saccharomyces cerevisiae* (DNATOPO_YEAST; NP_013335), and the plant cysteine proteinase inhibitor from *Arabidopsis thaliana* (CYSPROINH_ARATH; NP_850570). The protein from *A. thaliana* was used as an outgroup to root the tree. Scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position in the sequence. (C) The cloned cystatin cDNA from *S. domuncula* was used for transfection of *E. coli*. After treatment of the bacteria with IPTG, the protein (cystatin; lane a) was extracted and purified as described under Materials and methods. M, size markers.

comprises the characteristic cystatin domain with a value of $1.3e^{-09}$ (http://myhits.isb-sib.ch/cgi-bin/motif_scan; Fig. 3A).

An alignment of the sponge cystatin sequence with the next closely related mammalian sequences from pig (leukocyte cysteine proteinase inhibitor 1, stefin A8, stefin A5), cattle (stefin-C) and the above-mentioned human sequences, as well as the sequences from *Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae* and *Arabidopsis thaliana* was performed and a phylogenetic tree was constructed (Fig. 3B). In the phylogenetic tree, the sponge cystatin groups with the mammalian cystatins of type 1. The insect putative protein CG31016 (e=4.4), the putative protein from *C. elegans* (e=7.3), the yeast enzyme (e=0.24) and the plant cysteine proteinase inhibitor (e=0.84) are only very distantly related. The sponge sequence shows only low similarity to other types of cystatin; for example, the similarity to human cystatin C (CAA36497) has an e-value of >0.17 (not included in the tree).

The sponge cystatin was expressed in *E. coli*. After transfection of the bacteria and induction with IPTG, a 43 kDa protein (composed of the 10.8 kDa cystatin and the 32 kDa GST tag; Fig. 3C) was obtained and purified as described under Materials and methods. The GST-His tag of the recombinant fusion protein was obtained after cleavage with enterokinase. The purified 10 kDa protein was used for the studies (not shown).

Proteolytic activity of silicatein

One potent broad-spectrum inhibitor of cysteine proteinases is E-64. The inhibitory activity of E-64 is demonstrated by application of the zymogram technique. Cathepsin L at a concentration of 0.3 μg (2 mU) was loaded onto a gel containing casein. After size separation in the gel, incubation and staining with Coomassie Brilliant Blue, the zone that had been hydrolysed by cathepsin L became clear and visible (not shown here). In parallel, a spicule extract from *S. domuncula* was applied to the gel. After the gel was incubated and developed, a clear band with a size of about 25 kDa could be identified (Fig. 4 lane a), corresponding to the silicateins in the extract (Müller et al., 2005). We attribute the slight difference in the molecular size of the 27 kDa protein band obtained after SDS-PAGE (absence of casein) of extracts and the 25 kDa protein band seen in the zymogram (presence of casein) to the different separation conditions in the gels. If the size-fractionated proteins, obtained after separation by SDS-PAGE (in the presence of casein),

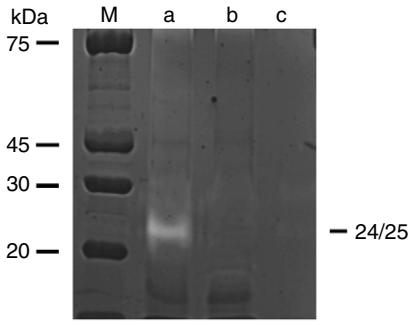

Fig. 4. Proteolytic activity of silicatein, present in spicule extract from *S. domuncula*. Extracts were prepared and separated by electrophoresis. After size separation the gel was incubated overnight at room temperature to identify the zones of proteolytic digestion as outlined under Materials and methods. The migration distance of the cleared zone (24/25 kDa) is characteristic for silicatein (lane a). If the sample from the spicules had been pre-incubated with E-64 (lane b) or with the recombinant sponge cystatin (lane c), no clearance zone could be seen. Form these data we conclude that silicatein, present in the spicule extract, still retains proteolytic activity. M, size markers.

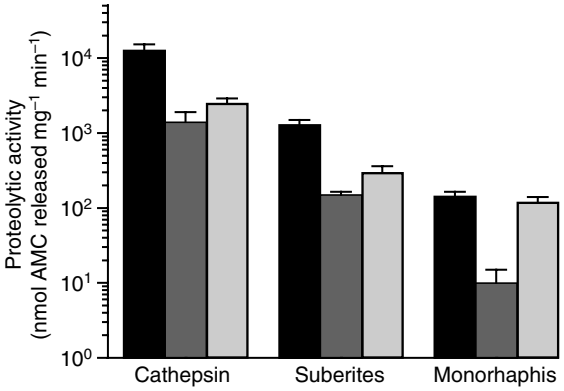

Fig. 5. Effect of cysteine proteinase inhibitors E-64 and cystatin on the proteolytic activity of cathepsin L (left), as well as spicule extracts from *S. domuncula* (middle) and *M. chuni* (right). The activity of the samples was determined either directly (black columns), or following pretreatment with E-64 (dark grey columns) or cystatin (light grey columns). The proteolytic activity was measured with the synthetic substrate Z-Phe-Arg-AMC, and is given as nmol AMC released mg^{-1} protein min^{-1}. Means (±s.d.) of five independent experiments are given.

were preincubated with E-64, the hydrolytic activity was completely abolished (Fig. 4 lane b). Likewise, the recombinant sponge cystatin was able to inhibit the proteolytic activity displayed by the *S. domuncula* spicule extract (Fig. 4 lane c). Consequently, we used these two inhibitors to characterize the proteolytic activity, extractable from the *M. chuni* extract.

Cathepsin-like activity in giant basal spicules

Total extracts from giant basal spicules were prepared and assayed for proteinase activity using Z-Phe-Arg-AMC as the substrate. The experiments were performed in parallel with *M. chuni* and *S. domuncula* spicule extracts to determine cathepsin L activity. The results showed that in this assay cathepsin L (concentration 300 ng per assay) had an activity of 12 800 nmol AMC released mg^{-1} protein min^{-1}, under the conditions used. If the inhibitor E-64 (1 μmol l^{-1}) was added, the activity dropped to values as low as

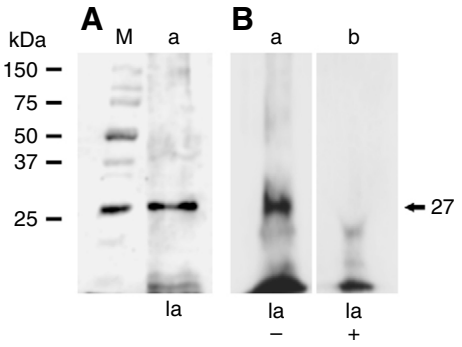

Fig. 6. Identification of the 27 kDa protein species in the total extract obtained from *M. chuni* giant basal spicules. (A) An extract from lamellae (la) of giant basal spicule was obtained and subjected to 10% SDS-PAGE. The 27 kDa protein is stained with Coomassie Brilliant Blue (lane a). M, size markers. (B) For the reaction with the labelled inhibitor (biotinylated E-64) the *Monorhaphis* spicule proteins were blot transferred and reacted with biotinylated E-64 as described under Materials and methods. The binding reaction with this reagent was performed either by direct addition of biotinylated E-64 to the blot (lane a, –; the 27 kDa protein is labelled) or after a pretreatment of the blot with a surplus of unlabelled E-64 (lane b, +).

1400 nmol AMC released mg^{-1} protein min^{-1}; if the sponge cystatin was added (5 μg ml^{-1}) the activity was likewise only 2450 nmol AMC released mg^{-1} protein min^{-1} (Fig. 5). The *S. domuncula* extract showed an activity of 1300 nmol AMC released mg^{-1} protein min^{-1}; the hydrolytic activity was almost totally blocked by both E-64 and cystatin (Fig. 5). The *Monorhaphis* spicule extract also displayed hydrolytic activity with 144 nmol AMC released mg^{-1} protein min^{-1}. However, the extract from this sponge was only inhibited by E-64 (by 85%), not by cystatin (Fig. 5).

Detection of the active site in the enzyme using labelled E-64 as a probe

Based on the finding that the natural inhibitor E-64 reduces the proteolytic activity in the extracts of *S. domuncula* and *M. chuni*, this inhibitor was used as a tool to identify the E-64 'binding protein'. The extract from *M. chuni* lamellae was size separated; the major band, corresponding to a protein of 27 kDa, could be visualized with Coomassie Brilliant Blue (Fig. 6A lane a). If the protein(s) were blot transferred and probed with the biotinylated E-64, it became apparent that only the 27 kDa molecule was recognized by the inhibitor (Fig. 6B lane a). However, if the blot was first incubated with a surplus of unlabelled E-64 and subsequently incubated with biotinylated E-64, no band could be observed (Fig. 6B lane b). From this result we conclude that the 27 kDa molecule corresponds to a silicatein-like molecule, sharing the molecular mass and also the binding affinity to E-64 with the demosponge silicateins.

DISCUSSION

M. chuni and *M. intermedia* are deep-sea sponges, which live in a milieu highly under-saturated in silicon (see Maldonado et al., 2005). Therefore, it is very surprising that in this habitat these two hexactinellid sponges synthesize the largest bio-silica structures existing on earth (see Introduction). From studies with the demosponge *S. domuncula* it is known that sponges have the potential to actively accumulate silicate, through a silica–bicarbonate {$Na^+/HCO_3^-[Si(OH)_4]$} cotransporter (Schröder et al., 2004). We have recently postulated that all siliceous sponges, including *Monorhaphis*, are provided with enzymatic machinery to polymerize/condense bio-silica through the enzyme silicatein (Müller et al., 2007b; Wang et al., 2007) to construct/synthesize their spicules, which may be up to 3 m long and 10 mm thick in the case of *Monorhaphis*. The data presented here support this assumption. For our studies either giant basal spicules or tauactins from *Monorhaphis* were used. These types of spicule were used in the earliest study by Schulze (Schulze, 1904) to identify and characterize the bio-silica material and to understand their growth. This author proposed that the lamellar, appositional thickening as well as the directional growth of the spicules is guided by organic filamentous sheets layered on their surface. The nature of these organic filaments remains to be determined; light (Schulze, 1904; Schulze, 1925) and electron microscopic studies identified filaments reminiscent of collagen around the spicules (Ehrlich and Worch, 2007; Müller et al., 2007b; Wang et al., 2007). The spicules contain about 10% (w/w) water and 4% (w/w) organic material (Schulze, 1904; Schulze, 1925). Even though, or perhaps because, the spicules (in the giant basal spicules of *Monorhaphis*) are composed of up to 400 lamellae and of organic material they can act as efficient optical glass fibres allowing light transmission (efficiency >60%) between wavelengths of 615 and 1310 nm [in *Hyalonema sieboldi* (Müller et al., 2006b)] or 600 and 1400 nm [in *M. intermedia* (Wang et al., 2007)]. Following the experimental

evidence and suggestions of Aizenberg et al. (Aizenberg et al., 2005) and Mayer (Mayer, 2005), the organic phases in the rigid bio-silica material provide the spicules with exceptional optical properties and high viscoelasticity. Etching experiments with HF, to partially convert bio-silica to gaseous silicon tetrafluoride (Monk et al., 1993), showed that the lamellae composing the bio-silica shell are not homogeneous; granules/spheres of approximately 100 nm in size are exposed (Müller et al., 2007c). A comparable finding has previously been documented using electron microscopy in demosponges (Pisera, 2003). This author also showed that in the outer lamellae the silica particles (40–300 nm) are arranged in a concentric and ordered string-like manner, reminiscent of collagen fibres. However, the diameters of the silica bundles are too big to correspond to organic fibres, which are around 20 nm for demosponge collagen (Schröder et al., 2006; Eckert et al., 2006).

In demosponges the silicateins exist both in the axial filament (Cha et al., 1999; Müller et al., 2005), which fills the axial canals, and on the surfaces of lamellae/layers, which are formed during the appositional growth of the spicules (Müller et al., 2005; Müller et al., 2006a). The spicules of the hexactinellids are also composed of lamellae, which allow the thickening growth of these skeletal elements (Schulze, 1904). In the centre of the spicules there exists the rectangular-shaped axial filament. To obtain an insight into the proteinaceous composition of the total spicules, including the axial canal, we compared the proteins from extracts of total spicules with those obtained from separated lamellae. An analysis of the composition of isolated axial filaments was not successful, since the axial filament (diameter of 1–5 μm) is small in comparison to the diameter of the total giant basal spicules (2–8.5·mm). SDS-PAGE analysis of the fractions revealed that in total extract of *M. chuni*/*M. intermedia* spicules a 27 kDa protein exists in addition to the 70 kDa molecule(s), while the organic component of the lamellae consists of only protein(s) of 27 kDa.

In an earlier study we obtained the first indications that total extract from giant basal spicules contains molecules related to silicatein (Müller et al., 2007b; Wang et al., 2007). In the present study we found that the total extract as well as the extract from separated lamellae of these spicules contains a silicatein(-related) molecule, with respect to size, post-translation modification and enzyme activity. SDS-PAGE showed that characteristic low-molecular mass protein(s) of 27 kDa exist in the spicules, which match in size with silicateins from demosponges (Cha et al., 1999; Krasko et al., 2000; Müller et al., 2007a) and correspond to the mature enzyme. As known from the different forms of the silicateins in *T. aurantium* (Cha et al., 1999) or *S. domuncula* (Krasko et al., 2000; Müller et al., 2003), these molecules are expressed/translated as a pro-enzyme (signal peptide–propeptide–mature enzyme: 36.3 kDa) and processed via the 34.7 kDa form (propeptide–mature enzyme) to the 23 kDa mature enzyme.

In the processed form the silicateins from *S. domuncula* have a size of ~27 kDa, if analysed by SDS-PAGE (Müller et al., 2005). It is likely that during the transport through the endoplasmic reticulum and the Golgi complex these molecules undergo post-translational modification. The silicateins either remain in vesicles where they form rods, the axial filaments, or are released into the extracellular space (Müller et al., 2005). In both compartments silicateins exist in five forms, characterized by pI values between 5.5 and 4.3 (Müller et al., 2005), suggesting stepwise phosphorylation of the molecules. As shown here, spicules from *Monorhaphis* also contain a set of 27 kDa proteins, which can be separated into five phosphorylated forms with pI values between 6.6 and 5.6 by two-dimensional gel electrophoretic analyses. The separation also showed that, besides the panel of 27 kDa proteins, an additional set of 30 kDa molecules exists comprising the same phosphorylation steps. A similar double-string pattern has been described for the silicateins from *S. domuncula* (Müller et al., 2005). Such a high resolution of the different forms of molecules could be achieved by application of a mild procedure for dissolution of the silica shell around the spicules (treatment with HF for only 3 h followed by an immediate step of dialysis against a Tris-HCl buffer). In contrast, if the dissolution process with HF was extended for 12 h, only protein molecules with a pI value of 6.6 could be detected (not shown), supporting the assumption that in the native state the spicule proteins exist as phospho-proteins (Kröger et al., 2002). This presumptive similarity between the demosponge silicatein and the hexactinellid protein was further strengthened by the finding that the 27 kDa *Monorhaphis* proteins cross-react immunologically with the demosponge silicatein.

The nature of the high-molecular mass proteins present in the total spicule extract from *Monorhaphis* has not yet been determined. Recent data suggested that in spicules of the hexactinellid *Hyalonema sieboldi*, collagen is the major protein (Ehrlich and Worch, 2007). However, additional chemical data supporting this conclusion have to be presented, at least for *Monorhaphis*. An interesting finding is that in *Monorhaphis* these proteins have been highly modified by charged groups, as reflected by the stepwise decrease/increase in the pI values. While one set of molecules is seen in a pI range between 4.7 and 4.2, the second set is characterized by pI values between 6.8 and 7.3. It might be speculated that the proteins are modified by differential phosphorylation and/or glutamylation or tyrosination, as described for the silica-depositing proteins in diatoms (reviewed in Perry, 2003). Further functional studies will solve the question of why these molecules exist only in the central region of the *Monorhaphis* giant basal spicules. The existence of fibrillar structures, visible after dissolution of silica by light microscopic analysis in the lateral lamellae, has been reported previously (Schulze, 1904; Schulze, 1925; Ehrlich and Worch, 2007). However, due to the applied extraction procedure in the presence of HF or alkali, the possibility cannot be excluded that these filaments are the result of an artificial aggregation. At present, we attribute these 70 kDa molecule(s) to the central core of the spicules, the cylinder around the axial canal, which is apparently composed of a more solid bio-silica shell.

Since silicateins belong to the class of cathepsin L enzymes, we approached in this study the potential proteolytic activity of the silicateins. In this series of experiments spicule extracts from *S. domuncula* and *Monorhaphis* were compared. The first hints that extracts from giant basal spicules of *Monorhaphis* display proteolytic activity came from a recent study (Müller et al., 2007b). It was shown that spicule proteins of >70 kDa in size show proteolytic activity in both sponges. Considering our findings indicating that the proteolytic activity of spicule extracts as well as the size of the molecules depend on the HF treatment procedure (W.E.G.M., A.B. and U.S., manuscript submitted), here we applied mild extraction procedures, as outlined above. The extraction conditions had been optimized for the *S. domuncula* spicule extract. The results obtained using the zymogram assay system indicated that the 24/25 kDa protein (corresponding to the size of silicatein) displays proteolytic activity. Next, using this technique we determined whether the proteolytic activity in the *S. domuncula* spicule extract can be affected by the inhibitors E-64 and cystatin. E-64, a well-established irreversible cysteine proteinase inhibitor (Barrett et al., 1982; Gour-Salin et al., 1994), interacts with the S_2 subsite (binding pocket) of the enzyme and blocks its binding to

bulky hydrophobic or aromatic residues of the inhibitor, e.g. to Phe in the P_2 position (Gour-Salin et al., 1994). Of particular interest are residues 133 and 157 (referring to papain numbering) in the enzyme, which form part of the binding pocket, and residue 205, which closes the end of the pocket (Brömme et al., 1994). In *S. domuncula* silicatein-α, the Ala-133 residue (corresponding to amino acid 249 in the sponge sequence) and also Leu-157 (amino acid 275; close to His in the catalytic triad) are highly conserved, like amino acid 205 (amino acid 325). This last residue can have an exchange between Glu and Ala (Brömme et al., 1994); Ala is found in silicatein (Müller et al., 2007a). E-64 was found to be a strong inhibitor of *S. domuncula* silicatein and also of the proteolytic activity measured in the *Monorhaphis* extract. As a substrate to detect the enzyme activity, the cathepsin L-specific synthetic dipeptide derivative Z-Phe-Arg-AMC was used (Mort, 2002). It was demonstrated that the proteolytic activity of the spicule extracts can be blocked by E-64 to over 90% at concentrations as low as 1 μmol l^{-1}. Based on this fact, we tested the possibility that biotinylated E-64 can be used as a probe to detect silicateins on blots after SDS-PAGE size separation. This attempt was based on earlier experiments which revealed that chemical modification of amino acids within E-64 does not qualitatively change the binding specificity of the inhibitor (Gour-Salin et al., 1994). For our studies we used EDC to bind biotin to a modified residue of E-64. The experiments revealed that the labelled E-64 bound strongly and specifically to the 27 kDa protein existing in the lamellar extract of *Monorhaphis*, after separation by SDS-PAGE. This binding could be blocked by pretreatment of the blot with unlabelled E-64.

As a further potential inhibitor of the (potential) silicateins, cystatin was used. The cystatins are naturally occurring cysteine proteinase inhibitors (see Kopitar-Jerala, 2006). We identified and cloned a cystatin from *S. domuncula*. The only form we could identify was the cDNA encoding the cystatin A/B-related polypeptide. It is interesting to note that these molecules exist only in sponges and the deuterostomian branch, but not in Protostomia. Cystatin A/B polypeptides are strong modulators of bone resorption in mammals, by preferentially inhibiting cathepsin K (Osawa et al., 2003; Laitala-Leinonen et al., 2006). The sponge recombinant cystatin was prepared and found to inhibit the *S. domuncula* silicatein but not the *Monorhaphis* 24/25 kDa protein (silicatein-like molecule). Now, further studies must be performed to identify specific inhibitors of the silicateins, especially in comparison with the cathepsins (ongoing study), in order to clarify the role of serine, present in the catalytic triad of silicateins (replacing cysteine), during proteinase cleavage.

Taken together, the data presented here reveal that the 24 kDa polypeptide in *Monorhaphis* has a series of characteristics in common with the silicateins found in demosponges; the size, the post-translational modifications and the proteinase activity. In addition, given that polyclonal antibodies directed against the demosponge silicatein cross-react with the *Monorhaphis* 27 kDa protein, the presence of such an enzyme appears to be highly likely. Furthermore, the cloning of the underlying gene is in progress. The demonstration of a silicatein-related protein in *Monorhaphis* in total spicules, including the axial filament and the lamellae, supports the view that the initial formation of bio-silica, the first lamella, and then the appositional growth of the spicules is directed by silicatein. This enzyme might act in *Monorhaphis* together with a lectin, as proposed in other studies (Müller et al., 2007b; Wang et al., 2007), and like in demosponges (Schröder et al., 2006) as inner and outer boundaries for the organic cylinders/sheets in which the bio-silica is formed. It is not understood why during this process of appositional growth the thickness of the lamellae remains almost constant. The next tasks for our studies with *Monorhaphis* will be (i) cloning of the gene encoding silicatein and analysis of the role of the recombinant protein during bio-silica formation and (ii) study of the additional fibrillar structures within and on the spicules. Besides being of general cell biological interest, the information on the silicatein-based formation of glass fibres is of prime biotechnological importance for the application of sponge spicules in (nano)optics (Wang and Wang, 2006; Schröder et al., 2007a; Schröder et al., 2007b).

Material was provided by Dr Lüter and Dr Bernhard (Museum für Naturkunde; D-10155 Berlin); the examined specimen originates from the collection of the Zoological Institute in Leipzig and is kept at the Museum für Naturkunde Berlin, Germany (ZMB Por 12700). This work was supported by grants from the European Commission, the Deutsche Forschungsgemeinschaft (Schr 277/9-2), the Bundesministerium für Bildung und Forschung Germany (project: Center of Excellence *BIOTEC*marin; 03F0414), the National Natural Science Foundation of China (grant no. 50402023), the Basic Scientific Research Program in China and the International Human Frontier Science Program (RG-333/96-M).

The cystatin sequence from *Suberites domuncula* reported here, [CYTA_SUBDO] *SUBDOCYTA* (accession number AM411124), has been deposited in the EMBL/GenBank data base.

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