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Cytotoxic and anticancer agents in mucus of *Galaxea fascicularis*: Purification and characterization

Faith Min Yee Fung,[1] Shinro Tachibana,[2] Loke Ming Chou,[3] and Jeak Ling Ding [1,4]

[1]Marine Biotechnology Laboratory, [3]Reef Ecology Laboratory, and [4]Bioscience Centre, School of Biological Sciences, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260; and [2]Department of Natural Products and Bioscience, Eisai Co. Ltd., 1-3 Tokodai 5-chome, Tsukuba-shi, Ibaraki 300-26, Japan

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Abstract. Various cytotoxic components derived from the mucus of the coral *Galaxea fascicularis* were investigated. Chromatography of the crude mucus extract through CM-Sepharose separated the components into 15 peaks, nine of which were found to express cytotoxic activity against rat liver cells. Trypan blue dye exclusion assay indicated that peaks P4, P5, and P8b induced the highest level of cytopathy in normal rat liver cells. P4 and P5 contained both proteinaceous and nonproteinaceous components and had heat-labile protease activity. The cytotoxins in P4 and P5 were nonproteinaceous. Further purification of P8b using a normal-phase silica column on HPLC yielded a nonproteinaceous and sugar-rich cytotoxin, S2. Mass spectrometry indicated S2 to be 742 Da in size. On incubation with *endo*-β-galactosidase, S2 lost more than 50% of its activity, suggesting that its active moiety is glycosylated. Further analysis of the carbohydrate composition revealed that S2 contained an N-linked sialylate glycan chain of NeuNAcα(2-6)Galβ(1-4)GlcNAc. Of the cancer cell lines tested with the mucus components, a vincristine-resistant murine leukemia cell (P388/VCR) was found to be most susceptible, with an LD_{50} of 0.3 μg/ml, thereby suggesting the potential of this coral mucus as a source of important compounds for anticancer drug screening and development.

Marine pharmacognosy has, over the last two to three decades, yielded a fascinating array of natural products with pronounced pharmacological activities, which in turn have prompted major screening efforts by academic institutions, pharmaceutical companies, and public health organizations, in the search for new drugs from the sea (Munro et al. 1987; Rinehart et al. 1993). Marine invertebrates have been found to produce a variety of chemically interesting and biologically significant secondary metabolites. Some of these metabolites serve as lead compounds for drug development and can be used as pharmacological tools for basic research in life sciences. Examples may be drawn from didemnin B extracted from tunicates (Rinehart and Geoer 1981) and bryostatin I derived from bryozoans (Pettit et al. 1982; Kraft 1989), both of which are undergoing clinical trials. Other novel compounds from soft corals, like sarcophytol A derived from *Sarcophyton glaucum* (Fujiki et al. 1989) and 9,11-secosterol from *Gersemia fruticosa* (Lopp et al. 1994) have offered promising leads for new cancer chemopreventive agents.

Galaxea fascicularis is a hermatypic scleractinian coral that is capable of surviving in turbid waters and dominates Indopacific reefs, sometimes to the exclusion of other corals (Veron 1986). In our primary screening of the mucus of *G. fascicularis*, the partially enriched mucus supernatant (MS) was found to elicit cytopathic effects initially on normal rat liver cells (BL8L). However, on subsequent inoculations of MS, the BL8L cells acquired a gradual resistance to the cytotoxic substance. On the other hand, transformed rat hepatoma cells succumbed to a single inoculation at half the dosage (Ding et al. 1994). MS was also found to be effective against human liver cancer cells, PLC/PRF/5, the human breast cancer cells, MCF-7 (Fung et al. 1995). This paper reports the purification and further characterization of the cytotoxic components of MS as well as their anticancer potential. As a follow-up to our earlier study, BL8L cells were used in this work to investigate the cytotoxic action of the purified components of MS. Both proteinaceous and nonproteinaceous bioactivities are found in MS. This paper focuses on the nonproteinaceous cytotoxic components of MS.

Correspondence to: J.L. Ding. Fax: 65-779-2486.

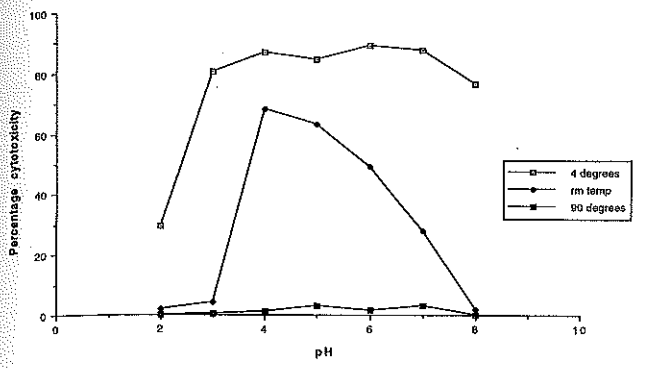

Fig. 1. pH and temperature optima profile of crude MS monitored against its cytotoxicity based on MTS assay on BL8L cells. Two sets of pH-adjusted MS were incubated for 24 h at 4°C and room temperature, while the third set was incubated at 90°C for 8 min before testing on BL8L cells.

Materials and methods

Preparation of mucus extract from G. fascicularis

The crude mucus extract, MS was prepared from *G. fascicularis* according to the method of Ding et al. (1994). The resulting MS was filter-sterilized through 0.22-μm filters and stored at −20°C for further purification and bioassays.

Cytotoxicity assays

Normal rat liver cells, BL8L (Manson and Green 1982) were cultured in phenol-red free Williams' E medium, supplemented with 10% foetal bovine serum, 10 mM HEPES, and 1% antibiotic–antimycotic (Gibco-BRL). The cells were incubated at 37°C in a humidified incubator supplied with 5% CO_2/95% air. To perform the cytotoxicity assay, 100-μl aliquots of 5.0×10^3 cells/ml were inoculated into each well of a 96-well plate. The cells were allowed to settle for 1 h at 37°C. Quadruplicate samples of MS or its purified components were added at increasing concentrations in 20-μl aliquots into the wells. The effects of MS against mitochondrial enzyme activity in BL8L cells were measured by adding equal volumes of a colorimetric cell proliferation test reagent, 0.2% MTS tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium–phenazine methosulfate (v/v: 20:1)] into each well. The cells were incubated for 1 h at 37°C, during which time MTS was converted into a formazan dye (Mosmann 1983). The absorbance of the reaction product was measured at $OD_{490\ nm}$. Correspondingly, the cell integrity was monitored by trypan blue dye exclusion test.

The secondary screening of MS was carried out on various tumour cell lines, namely human lung carcinoma WIDR; human colon cancer A549; human stomach carcinoma MKN-28; four human hepatoma cell lines, viz., HuH1, HuH2, Chang, and PLC/PRF/5; murine leukemia P388; and a multiple-drug-resistant murine leukemia cell line P388/VCR (derived by Eisai Co. Ltd., Tsukuba Research Laboratories). Normal human embryonic lung WI38 cells were also used as control. This assay was based on the growth-inhibiting properties of MS against the cancer cells over a period of 3 days.

Purification of cytotoxic component(s)

pH and temperature optima. Aliquots of MS were adjusted to pHs ranging from 2 to 8 with addition of 0.1 N HCl or 0.1 N NaOH. Two replicate series of pH-adjusted MS were incubated for 24 h either at 4°C or at room temperature. A third series of pH-adjusted samples was incubated at 90°C for 8 min and snap-cooled in ice. After incubation, cytotoxicity assay on BL8L was performed using 0.52 mg dry weight/ml. This was equivalent to the previously established LD_{50} of the crude MS on BL8L cells (unpublished data).

Ion-exchange chromatography. The purification protocol was established to selectively isolate cytotoxins that were stable at low pH. Initial adjustment of crude MS to pH below 3 yielded precipitates. The resultant precipitate was removed by pelleting at 1380*g*. Prescreening of the supernatant (pH3sup) and pellet (pH3pell) found the bioactive components to be retained in the supernatant. Thus, prior to column chromatography, MS was adjusted to pH 3 using 0.1 N HCl and the pH3sup was applied to a column (3.0 × 30 cm) of CM-Sepharose (Pharmacia), which was previously equilibrated with 25 mM sodium citrate, pH 3.5. The column was washed with six column volumes of the initial equilibration buffer and eluted with a linear gradient of 25 mM sodium citrate, pH 3.5, to 100 mM sodium phosphate, pH 6.5, containing 1 M NaCl. Fractions of 12 ml were collected and pooled as peaks according to absorbance at 280 nm. Active fractions were dialyzed by ultrafiltration in an Amicon chamber (Amicon, Beverly, MA, USA) equipped with a YM10 Diaflo ultrafiltration membrane (Amicon). Unless otherwise stated, all procedures were carried out at 4°C.

HPLC fractionation. The most cytotoxic peak, P8b, from the CM-Sepharose column was dissolved in 60% acetonitrile, and 100-μl aliquots of the solution containing 0.36 mg dry weight of P8b were chromatographed through a 4.6 × 250 mm Spherisorb Silica column (Phenomenex) that was equilibrated with 100% acetonitrile. The elution was initially isocratic, using 100% acetonitrile for 15 min, followed by a gradual linear gradient of 100% acetonitrile to 75% acetonitrile for 45 min at a flow rate of 1 ml/min. The eluants were monitored by UV absorption at 205 nm. One-milliliter fractions were collected and speed vacuumed to dryness.

Characterization of cytotoxic components

SDS-PAGE. Reducing conditions of SDS–polyacrylamide gel electrophoresis were carried out according to the method of Laemmli (1970). Electrophoresed gels were stained in Coomassie brilliant blue (Wilson 1983) or by an improved silver staining procedure (Wray et al. 1981).

Protease activity. This assay was performed to assess the possible proteolytic mechanism by which crude MS and its purified peaks bring about cytopathic effects on cultured cells. The presence of proteolytic activity in crude MS, P4, P5, and P8b was tested using bovine serum albumin (BSA, fraction V, Sigma) as substrate. A total of 150 μg dry weight of each sample was added to 12.5 μl of 0.1 M sodium phosphate, pH 7.4, containing 25 μg BSA. The mixtures were incubated at 37°C for 2 h. In a separate experiment, the heat lability of the proteolytic activity in these samples was tested by preheating at 65°C for 2 h, followed by their reaction with BSA. The resultant products were analyzed on reducing SDS-PAGE.

Proteinase K digestion. Proteinase K from *Tritirachium album* (Sigma) was reconstituted to give a stock solution of 1 mg/ml. The amount of proteinase K added to individual test samples was equivalent to the total protein in each of the samples. Replicates of the resulting mixtures were incubated at 55°C for 2 h. The digestion was terminated by addition of 1 N HCl. The resultant products were analyzed by SDS-PAGE.

Thin-layer chromatography. TLC was used to resolve the nonproteinaceous components of P4, P5, P8b, and S2 that were not detectable by silver staining on SDS-PAGE gels. HPTLC plates (10 × 10 cm) precoated with Cellulose F (Merck, Darmstadt, Germany) were used. Samples of 2 μl each were applied and immediately dried with an air gun so as to keep the initial spot size within 4 mm diameter. A mobile phase consisting of propanol–acetic acid–water (3:3:2) was used, and the plate was dried before development in aniline–diphenylamine–phosphoric acid reagent. The reagent was prepared by mixing 2 g diphenylamine, 2 ml aniline, and 80 ml acetone, prior to dropwise addition of 15 ml phosphoric acid to the mixture. The spots were revealed after heating the plate to 85–120°C for 10–15 min. A duplicate set of TLC was sprayed with 0.2% ninhydrin in acetone and heated to 85–120°C, to reveal the presence of free amino acids.

endo-β-*Galactosidase digestion and fluorescent detection of derivatised glycans.* *endo*-β-Galactosidase from *Escherichia freundii* (Seikagaku, Japan) was reconstituted in 10 μM sodium acetate buffer, pH 5.8, to give a stock concentration of 1 mU/μl. Aliquots of each sample

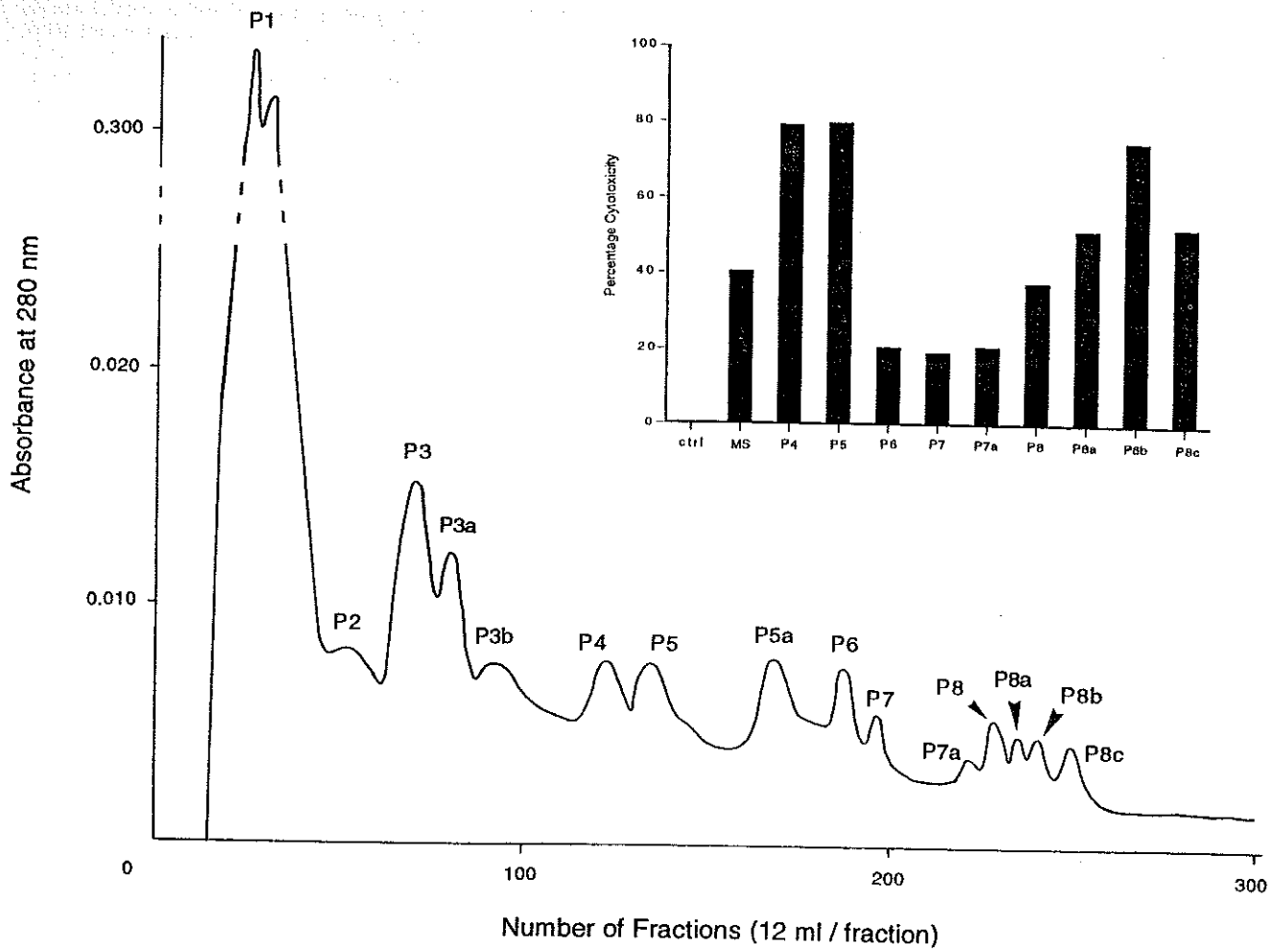

Fig. 2. Cation-exchange chromatography of crude MS using CM-Sepharose column (3.0 × 30 cm). Initial isocratic elution of the void peak (P1) was effected with 25 mM sodium citrate, pH 3.5, after which a linear gradient was used from 2.5 l of 25 mM sodium citrate, pH 3.5 to 2.5 l of 100 mM sodium phosphate, pH 6.5 with 1 M NaCl, at a flow rate of 2 ml/min. Inset shows cytotoxicity of MS and its purified peaks on BL8L cells using trypan blue dye exclusion assay. BL8L cells incubated with MS or its components for 16 h at 37°C were rapidly trypsinized and resuspended in 0.4% (w/v) trypan blue dye. The percentage cytotoxicity of each sample was calculated as the number of nonviable cells over the total number of cells.

Table 1. Purification of MS.[a]

Purification step	Total dry wt (mg)	LD_{50} (mg/ml)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)
Crude MS	715.00	0.520	1375	1.9	1.0
CM-Sepharose					
P4	27.20	0.340	80	2.9	1.5
P5	51.30	0.320	160	3.1	1.6
P8b	85.00	0.210	405	4.8	2.5
HPLC Silica					
S2	5.88	0.026	226	38.4	20.2

[a] Purification of crude MS through CM-Sepharose and Spherisorb silica column. An LD_{50} unit was established as the amount of test sample needed to bring about 50% cell death in BL8L cells. Total activity in units was calculated from the ratio of total dry weight (mg) to LD_{50} (mg/ml).

equivalent to three times the LD_{50} was reacted with 30 mU enzyme for 2.5 h at 37°C. Controls consist of 30 mU enzyme incubated for 2.5 h at 37°C with equal volume of the sample buffer. The resultant test samples and controls were used to analyze for the loss of cytotoxicity against BL8L cells.

The degree of glycosidase digestion of the samples were monitored by 2-aminobenzamide fluorescent labeling (Signal 2-AB, Oxford GlycoSystems Ltd.). The conjugation with 2-AB proceeds according to a standard reduction amination reaction for a glycan carrying *N*-acetylglucosamine at its reducing terminus. The derivatized glycans were chromatographed on HPLC using a Carbohydrate Analysis column (WAT084038, Waters) equilibrated with 100% acetonitrile. The samples were eluted with an increasing water gradient of up to 100% water and monitored at 254 nm.

Glycan differentiation with digoxigenin-labeled lectins. DIG glycan differentiation kit (Boehringer Mannheim Biochemica) contains digoxigenin-labeled lectins [*Galanthus nivalis* agglutinin (GNA), *Sambucus*

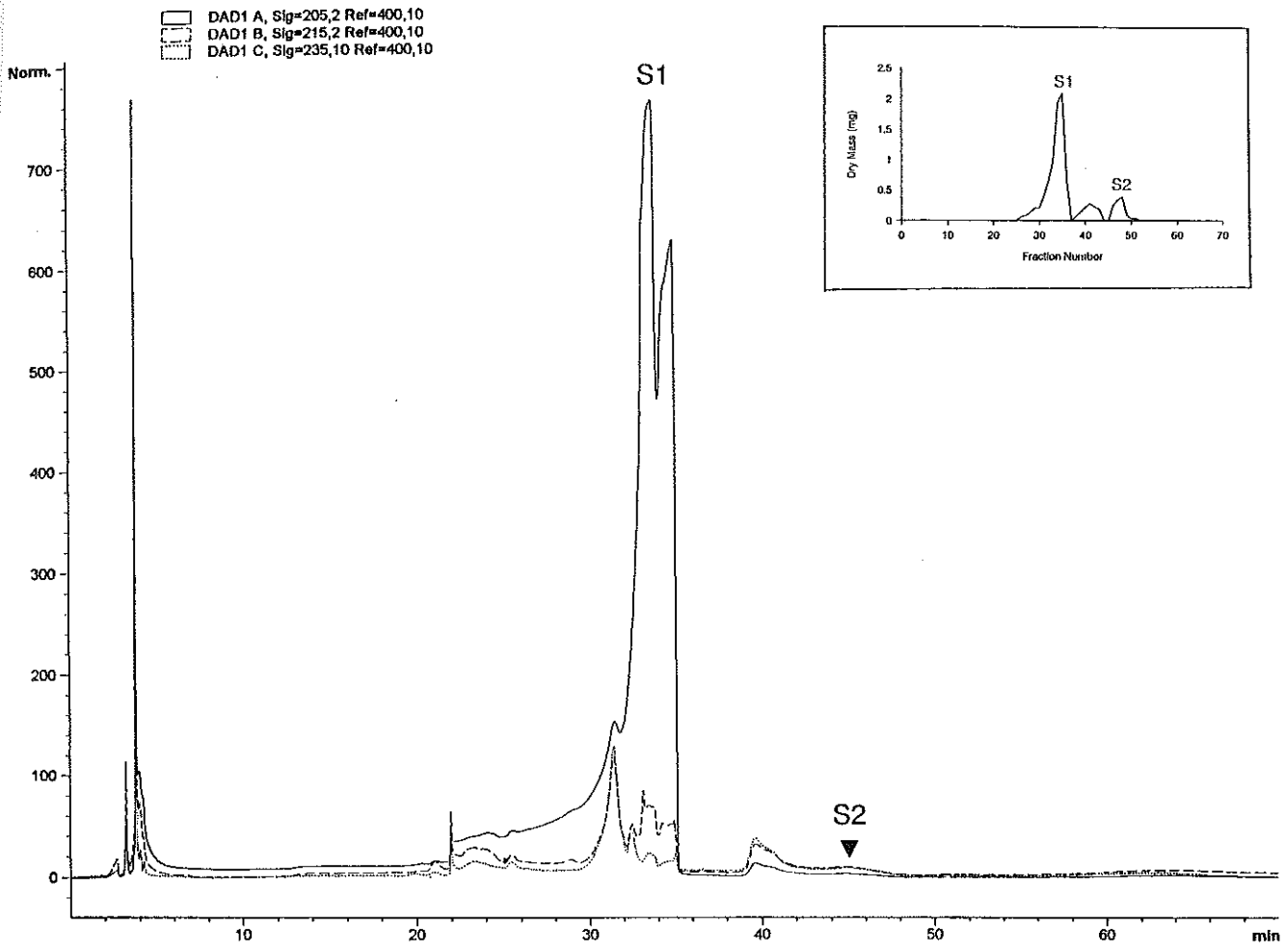

Fig. 3. Normal-phase HPLC of P8b through Spherisorb silica column. An isocratic elution of 100% acetonitrile was applied for 15 min, followed by a linear gradient from 100% acetonitrile to 75:25 acetonitrile–water mix for 45 min, at a flow rate of 1 ml/min. The elution profile was monitored at $OD_{205\ nm}$ (——), $OD_{215\ nm}$ (------), and $OD_{235\ nm}$ (. . . .). Inset shows the monitoring of the elution profile by dry mass.

nigra agglutinin (SNA), *Maackia amurensis* agglutinin (MAA), peanut agglutinin (PNA), and *Datura stramonium* agglutinin (DSA)]. It utilizes the specific binding of the lectins to carbohydrate moieties and the identification of these structures via immunological detection of the bound lectins. Briefly, samples of 0.1 mg each were spot wetted onto an Ultrabind membrane (Gelman Sciences) and allowed to air-dry. The membrane was then incubated overnight at room temperature in blocking solution. After washing twice in phosphate-buffered saline (PBS), the membrane was incubated with the respective lectin solution for 1 h, followed by another round of washing. The membrane was further incubated in anti-digoxigenin-AP for 1 h and washed again in PBS. Finally, the membrane was developed in a staining solution of NBT/BCIP until the desired color development was obtained.

Results and discussion

Purification of cytotoxic principles

The nonproteinaceous cytotoxins were purified from the crude mucus sample, MS, by ion-exchange chromatography followed by normal phase HPLC. Although the physiological pH of MS was between 7 and 7.5, the cytotoxic activity in crude MS was most stable at pH 3–4 when maintained at 4°C (Fig. 1).

Nine cytotoxic fractions derived from ion-exchange chromatography of MS

Adjustment of MS to pH 3 aided the removal of precipitates without significant loss in nonproteinaceous cytotoxicity. The resultant supernatant, pH3sup was thus partially enriched. Chromatography through CM-Sepharose resolved pH3sup into 15 peaks (Fig. 2). Nine of the 15 peaks expressed cytotoxicity against BL8L cells, of which P8b was most potent, giving a 2.5-fold increase in specific cytotoxic activity (Table 1). The trypan blue dye exclusion test of all nine peaks revealed that P4, P5, and P8b showed highest cytoxicity against BL8L cells (inset to Fig. 2). A study on the combined cytotoxicity of P4, P5, and P8b showed additive, but not synergistic effects. P8b was further purified by HPLC.

HPLC Purification of P8b yielded a 742-Da cytotoxin

Passage of P8b through a Spherisorb silica column, monitored at 205 nm, gave a major joint peak between tubes 30 and 35 (Fig. 3). When correlated against dry weight of each

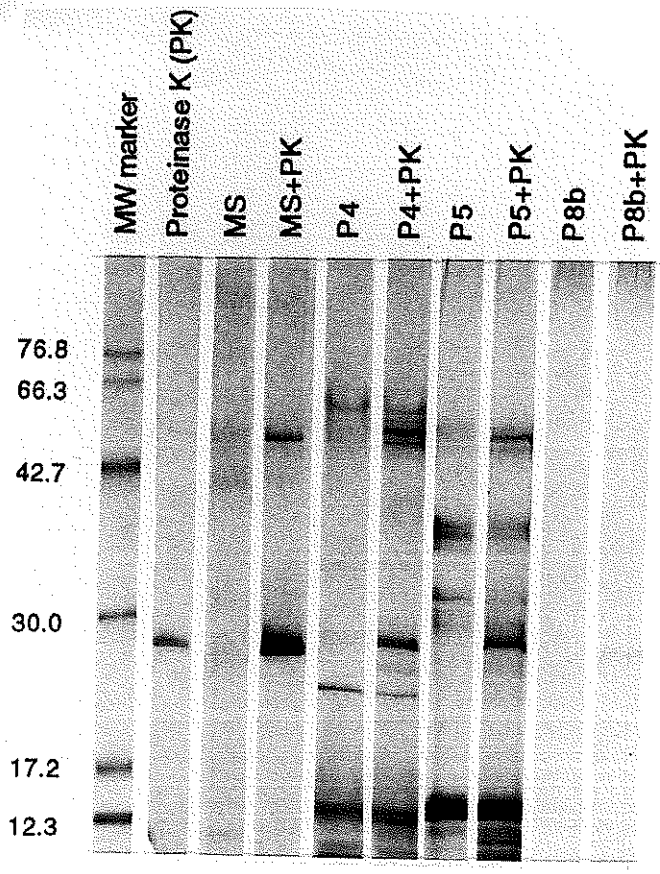

Fig. 4. SDS-PAGE analysis of crude MS, P4, P5, and P8b before and after proteinase K (PK) digestion. The amount of proteinase K inoculated into each sample was equivalent to the total protein content of individual fractions. The samples were digested for 2 h at 55°C and electrophoresed on a gradient of 10–20% SDS-PAGE gel.

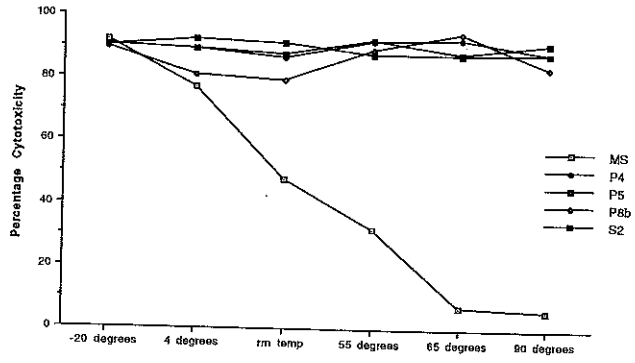

Fig. 5. The heat lability of the cytotoxins in MS, P4, P5, P8b, and S2. Samples were incubated at 4°C and room temperature (23°C) for 24 h before testing their resulting cytotoxicity on BL8L cells. The heating of the samples at 55°C and 65°C was carried out for 2 h, while the treatment of samples at 90°C was performed for only 8 min. Another replicate of samples stored at −20°C was similarly tested.

fraction, one major and two minor peaks were evident (see inset, Fig. 3). The two minor peaks absorbed minimally at 205 nm. Hence, all the fractions were collected and speed-vacuumed to dryness. The peaks were pooled separately and tested for cytotoxicity on BL8L cells. S2 was most cytotoxic, giving 20-fold increase in specific cytotoxic activity (Table 1). Preliminary LC-mass spectrometry of S2 using Perkin Elmer Sciex suggests a molecular weight of 742. In view of the earlier observation that the cytotoxin was larger than 10 kDa, S2, which is only 742 Da, is probably previously associated with a larger molecular component.

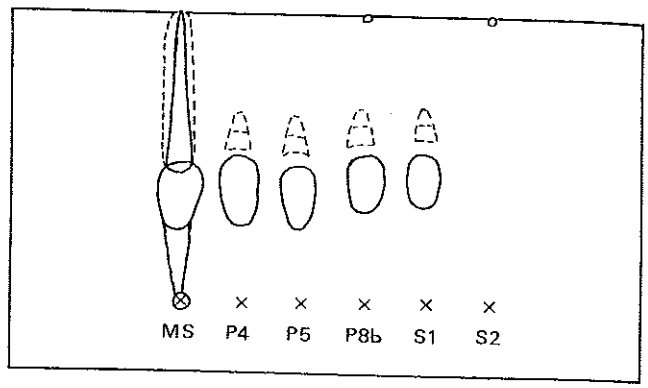

Fig. 6. Thin-layer chromatography of crude MS and its constituents on cellulose-coated HPTLC plates developed with propanol–acetic acid–water (3:3:2). Two microliters of each sample was spotted and the resolved components were revealed by aniline–diphenylamine–phosphoric acid reagent (——) and ninhydrin (------).

Characterization of bioactivity in MS

The biochemical properties, cytopathic activities, and anti-cancer potentials were determined in the bioactive peaks purified from MS.

Cytotoxins in P4, P5, and P8b are nonproteinaceous

Silver staining of SDS-PAGE showed the three most cytotoxic peaks P4, P5, and P8b were unique one from another. P4 and P5 contained different patterns of protein bands ranging in size from 17 to 73 kDa. In contrast, P8b did not show any protein bands (Fig. 4). Although SDS-PAGE analysis of proteinase K-digested P4 and P5 showed altered banding patterns, these two peaks retained their cytotoxicity, thereby suggesting that their bioactive components are either nonproteinaceous or proteinase K resistant. The cytotoxicity of P8b also remained unaffected by proteinase K treatment, thus confirming its nonproteinaceous nature. However, crude MS digested with proteinase K resulted in diminished cytotoxic activity, therefore indicating that MS contains a multiple system of cytotoxins, where the proteinaceous cytotoxins could have been removed in the pH 3 precipitate prior to ion-exchange chromatography. Thus, the nonproteinaceous cytotoxins are represented in P4, P5, P8b, and subsequently, in S2.

Cytotoxic activities in P4, P5, P8b, and S2 are heat-resistant

Peaks P4, P5, P8b, and S2 still retained their cytotoxic activity even when incubated at increasing temperatures of up to 90°C for 8 min, while crude MS appeared to be heat-labile, losing 50% of its activity when incubated at room temperature for 24 h (Fig. 5). This confirms that MS contains a system of multiple cytotoxins, constituted by both heat-labile proteinaceous and heat-resistant nonproteinaceous components.

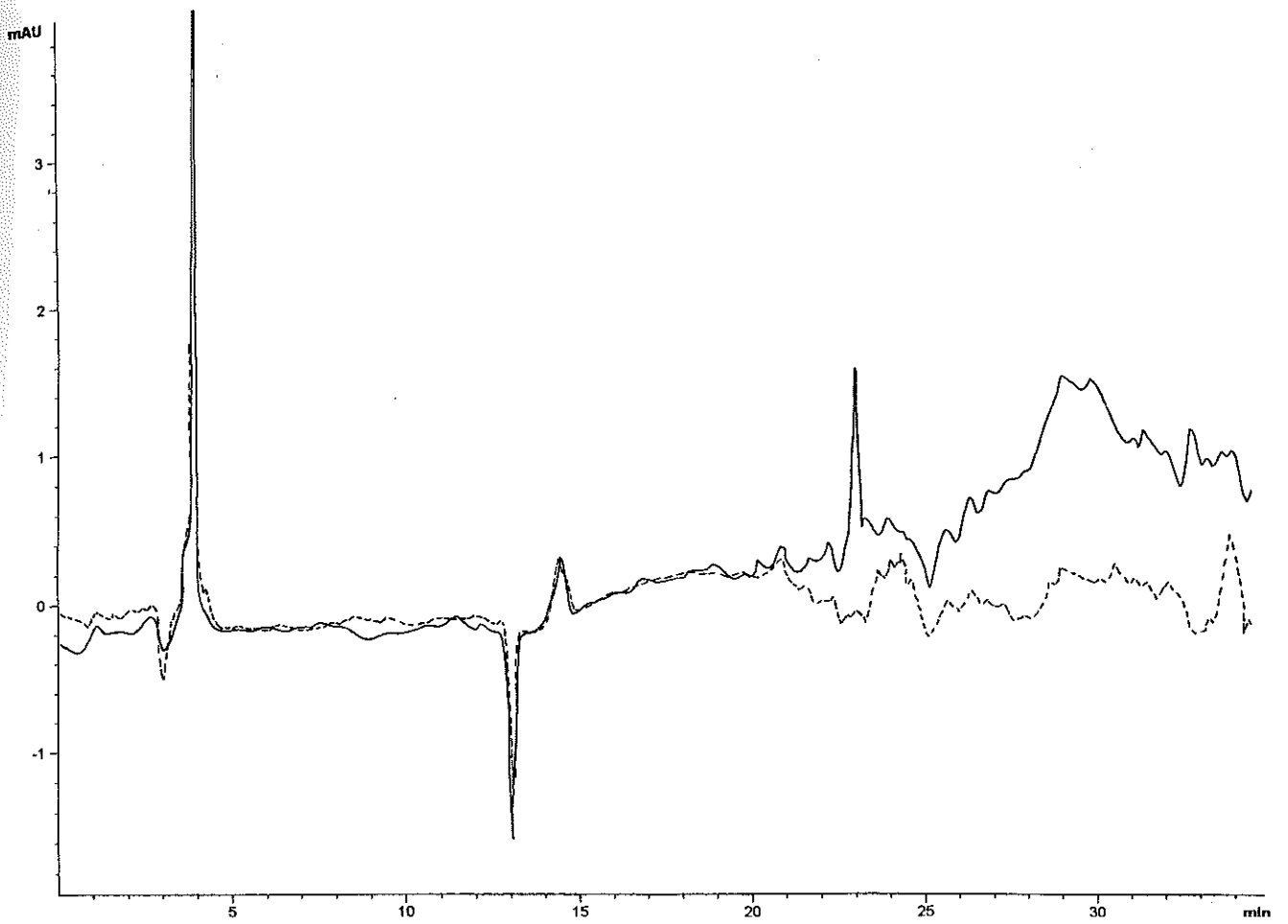

Fig. 7. HPLC elution profile of *endo*-β-galactosidase-digested S2 tagged with 2-AB signal label. S2 control (——) and enzyme-digested S2 (------) were fluorescent-tagged with 2-AB and chromatographed using Waters carbohydrate analysis column. The bound fluorescent product was eluted with a linear gradient from 100% acetonitrile to 100% water.

P4, P5, P8b, and S2 contain sugar moieties

TLC (Fig. 6) resolved P4 and P5 into two components, one of which was detected by ninhydrin (R_f 0.72) and the other by a Schiff's-based reaction using aniline–diphenylamine–phosphoric acid reagent (R_f: 0.42 for P4 and 0.39 for P5). On the other hand, P8b was resolved into three components, two of which were detected by the Schiff's reagent giving R_fs of 0.44 and 1.0, and the third by ninhydrin (R_f 0.72). S1 and S2 appeared to have been enriched with sugar moeities derived from P8b, with a single glycosylated spot each at 0.44 for S1 and 1.0 for S2. The sugar-containing groups from P4 and P5 showed slower migration than those of P8b and S1, thus again indicating the different nature of P4 and P5 from P8b.

Glycosylated moieties in P4, P5, P8b, S1, and S2 are responsible for their cytoxicity

Incubation of P4, P5, and P8b with *endo*-β-galactosidase led to a loss of 40–55% of cytotoxicity. S1 and S2, derived from P8b, exhibited losses of 45% and 52% of cytotoxicity, respectively (data not shown). Pretreatment of S2 with *endo*-β-galactosidase resulted in cleavage of the *O*-glycosylated sugar moieties whereby 2-aminobenzamide labeling of the digested fragments showed a loss in UV absorbance at 23–35 min of the elution profile (Fig. 7). Therefore, the loss of cytotoxicity following *endo*-β-galactoside digestion indicates the involvement of sugar moieties in the active site(s) of the cytotoxic molecule.

Sugar composition of P4, P5, P8b, and S2

Digoxigenin-labeled lectins bound specifically to different glycans in P4, P5, P8b, and S2 (Fig. 8a). P4 was probably heterogenous, as it was able to bind to all the test lectins, albeit with special affinity for MAA. This indicates the dominant presence of terminal sialic acid linked α(2-3) to galactose. On the other hand, P5 binds strongly to GNA and slightly to SNA and DSA. This suggests that P5 is comprised largely of *N*-glycosidically linked hybrid-type carbohydrate chains, characterized by a common pentasaccharide, trimannosyl core ($Man_3GlcNAc_2$) with terminal sialic acid linked α(2-6) to galactose (Fig. 8b). P8b showed similar characteristics to S2, with the exception of a strong affinity for GNA, indicating a high mannose composition that is lacking in S2.

The susceptibility of S2 to *endo*-β-galactosidase, leading to the loss of 50% of its cytotoxicity suggests the presence of Galβ(1-4)GlcNAc linkage that is critical for its bioactiv-

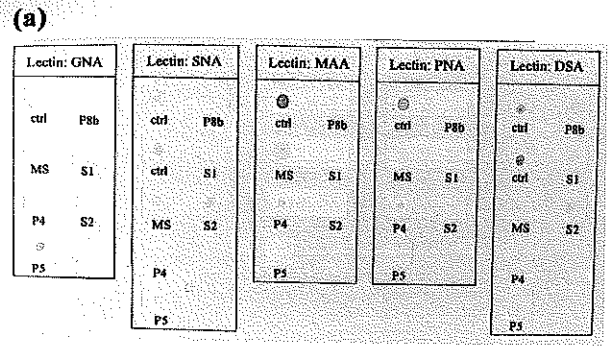

Fig. 8. Determination of sugar components in P4, P5, P8b, and S2 using digoxigenin-labeled lectins. **(a)** dot blot of MS and its purified components, together with positive glycan controls, incubated with different lectins; and **(b)** proposed structures of P5 and S2 derived from DIG glycan differentiation study. The lectins and enzymes directly involved in the derivatization of the structure are presented in bold.

ity. The presence of such sugar residues in S2 is further corroborated by its strong affinity for both DSA and SNA, which indicates the presence of Galβ(1-4)GlcNAc and sialic acid terminated α(2-6)Gal, respectively, Taken together, we propose that S2 contains a sialylated *N*-linked glycan chain comprising NeuNAcα(2-6)Galβ(1-4)GlcNAc (Fig. 8b). The glycosylated moiety would constitute a molecular weight of 675 Da, which again agrees closely with the molecular weight of 742 Da of the entire S2 molecule previously determined by mass spectrometry.

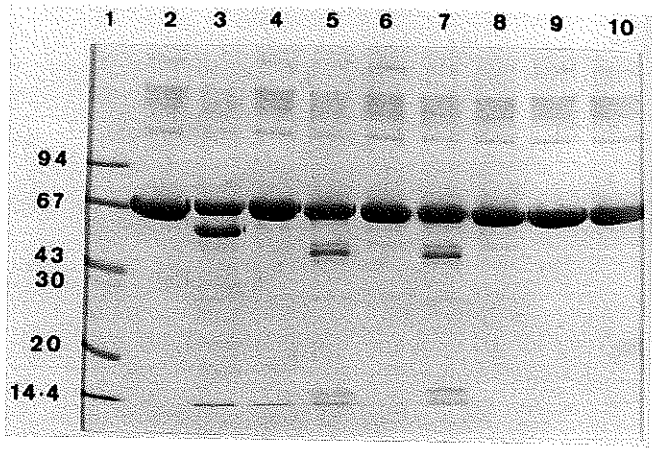

Fig. 9. Presence of protease activity in crude MS and its constituents. Test samples were incubated with BSA as substrate in a ratio of 6:1 at 65°C for 2 h before electrophoresis. Undigested control BSA, incubated under the same conditions with equal volumes of buffer remained intact. Lane 1: low-molecular-weight markers (Pharmacia); 2: BSA; 3: BSA + MS; 4: BSA + heated MS; 5: BSA + P4; 6: BSA + heated P4; 7: BSA + P5; 8: BSA + heated P5; 9: BSA + P8b; 10: BSA + heated P8b.

Proteolytic activity in P4 and P5 does not contribute to its cytotoxicity

Crude MS appeared to proteolytically digest BSA to release a high-intensity protein band at 58 kDa, while P4 and P5 also digested BSA to release a major fragment of 48 kDa (Fig. 9). On the other hand, P8b did not digest BSA at all. Heat treatment of MS, P4, and P5 at 65°C for 2 h led to complete loss in their proteolytic activity towards BSA. Since the cytotoxic action in P4 and P5 was found to be heat-resistant (Fig. 5), the heat-labile proteolytic activity in P4 and P5 should not contribute directly to the observed cytotoxicity on BL8L cells. Peak P8b, which lacks proteolytic activity, probably acts via a different cytotoxic mechanism.

Cytopathy of P4 and P5 on BL8L cells is unique from P8b

Morphological observations of the cytopathic effects of P4 and P5 monitored over 16 h showed rounding up of BL8L cells, which later became detached from the culture dish, followed by eventual cell death. In contrast, P8b brought about enucleation, intense shrinkage and, finally, fragmentation of the whole cell. Hence, the cytopathy effected by P4 and P5 on BL8L cells should be different from that of P8b.

MS is cytotoxic to a multiple-drug-resistant leukemia cell line, P388/VCR

Normal fetal lung cells, WI38, remained unaffected by MS at concentrations up to 0.8 mg/ml (Table 2). All the cancer cells tested showed slight susceptibility to MS except for HuH2 cells, which exhibited a moderate LD_{50} of 0.13 mg/ml, while a multiple-drug-resistant murine leukemia cell line, P388/VCR, was particularly susceptible, succumbing with an LD_{50} of 19.2 μg/ml. P388/VCR, which is resistant to a well-established cancer chemotherapeutic drug, vincris-

Table 2. Anticancer screening of crude MS against various cancer cell lines.[a]

Cell Line	Description	LD_{50} (mg/ml)
WI38	Human normal fetal lung	>0.80
WIDR	Human lung carcinoma	>0.80
A549	Human colon cancer	>0.80
MKN-28	Human stomach carcinoma	>0.80
PLC/PRF/5	Human hepatoma	0.73
HuH1	Human hepatoma	0.64
HuH2	Human hepatoma	0.13
Chang	Human hepatoma	>0.80
P388	Murine leukemia	0.094
P388/VCR	Murine multiple-drug-resistant leukemia	0.019

[a] Secondary anticancer screening assay on crude MS. Increasing amounts of MS was inoculated into 10 different cell lines and incubated at 37°C for three days before carrying out MTS assay to determine growth inhibition properties. With the exception of Chang cell line, all other cell lines with LD_{50} >0.80 mg/ml were not affected by MS cytotoxicity.

Table 3. Anticancer assay of purified peaks against P388 and P388/VCR.

Purification step	LD_{50} against P388 (μg/ml)	LD_{50} against P388/VCR (μg/ml)
Crude MS	96.0	19.2
CM-Sepharose		
P4	12.7	12.7
P5	35.5	17.7
P8b	7.8	5.8
HPLC Silica		
S2	0.6	0.3

[a] Anticancer screening of MS, P4, P5, P8b, and S2 on P388 and P388/VCR leukemia cell lines. An LD_{50} unit is the amount of test sample required to bring about 50% cell growth inhibition over a period of three days.

tine, was found here to be susceptible to crude MS. On the other hand, MS was five times less cytotoxic to the noninduced P388 leukemia cell line.

Anticancer screens using P4, P5, P8b, and S2 on both P388 and P388/VCR cells found P8b and S2 to closely mimic the action of crude MS on the two cell lines. They are more selective against the vincristine-resistant P388/VCR leukemia cell line than its non drug-resistant counterpart, P388 leukemia cell (Table 3). Compared to P4 and P5, P8b was more effective against P388 and P388/VCR, with LD_{50}s of 7.8 μg/ml on P388 and 5.8 μg/ml on P388/VCR. On the other hand, S2 was effective against P388 cells with an LD_{50} of 0.6 μg/ml, and even more potent against P388/VCR, with an LD_{50} of 0.3 μg/ml. Hence, S2 could be a potential anticancer candidate for further in vivo screenings.

Conclusion

The mucus of the coral *Galaxea fascicularis* consists of a system of multiple cytotoxins, some of which are heat-labile and proteinaceous, while others are heat-resistant, nonproteinaceous, and richly glycosylated. Of the nonproteinaceous components studied, peak P8b purified from passage of crude MS through CM-Sepharose showed the highest cytotoxicity against BL8L cells, compared to the other 14 peaks. Further purification of P8b through a HPLC silica column yielded S2, a cytotoxin of 742 Da. The active moeity of S2 is glycosylated, with a sialylated *N*-linked glycan chain, comprising NeuNAcα(2-6)Galβ(1-4)GlcNAc of 675 Da, which probably constitutes the active center of the cytotoxin, since its digestion with *endo*-β-galactosidase led to a drastic fall in its cytotoxicity. In a secondary anticancer screen, S2 was found to be most effective against a multiple-drug-resistant cell line, P388/VCR, with an LD_{50} of 0.3 μg/ml, thereby suggesting the feasibility of using this compound as a lead for anticancer agents.

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