Immunochemical Studies of Starfish Gangliosides: Production of Monoclonal Antibody against AG−2, the Major Ganglioside of Starfish *Acanthaster planci*, and Detecting Its Distribution in Tissues by TLC Immunostaining

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Abstract In this study, we establish a thin-layer chromatography (TLC) immunostaining method for detecting starfish gangliosides. A new monoclonal antibody (MAb) against AG-2, the major gangliosides molecular species of *Acanthaster planci*, was produced by fusing hybridoma with splenocytes immunized to liposomal AG-2. BALB/c male mice were injected with liposomal AG-2 antigen, and immunized. Their splenocytos were isolated and fused with hypoxanthine-aminopterine-thimidine (HAT)-sensitive mouse myeloma cells. Hybridomas producing MAb reactive to AG-2 were cloned using the limited dilution method. Established hybridomas were cultured in eRDF medium. Crude MAb produced from clone 8D4 was purified with a magnesium pyrophosphate column. Enzyme immunoassay and TLC immunostaining of AG-2 were performed using the purified MAb. Structurally related gangliosides did not cross-react with anti-AG-2 antibodies. The detection limit of TLC immunostaining was 50 ng of AG-2. The newly established immunostaining method was further developed for detecting AG-2 distribution and qualitative analysis in tissues and/ or organs. Our results show that the majority of AG-2 is present in the stomach of male *A. planci*, while AG-2 is distributed not only in the stomach but also in the the pyloric caeca of female *A. planci*.

Key words : Monoclonal antibody, *Acanthaster planci*, acanthaganglioside, AG-2 TLC immunostaining

Introduction

The immunoassay system using monoclonal antibodies (MAbs) is indispensable in biological investigation. However, since MAbs to naturally occurring bioactive compounds with low molecular weights are relatively rare, we have prepared MAbs and established enzyme linked immunosorbent assay (ELISA) systems for forskolin [14], marijuana compound [20], opium alkaloids [17], solamargine [6], ginsenoside Rb_1 [19], crocin [23] and glycyrrhizin [21]. Furthermore, an Eastern blotting method against ginseng saponins [3] and glycyrrhizin [16] was established during the search for natural resources and breeding of medicinal plants.

We have additionally examined biologically active glycosphingolipids (GSLs) from marine invertebrates [1,5,24]. SialoGSLs are well-known as gangliosides that were originally identified in the nervous tissue of vertebrates. Vertebrate gangliosides exist in the plasma membrane, and function as receptors for cholera toxin B [15] and influenza viruses [18]. On the other hand, among invertebrates, gangliosides are present only in echinoderms (sea urchin, starfish, and sea cucumber), and some display neuritogenic activity against rat pheochromocytoma PC 12 cells [4,10]. The physiological functions of gangliosides generally remain to be elucidated, except that of M5, the major ganglioside of sea urchin *Anthocidaris crassispina* [9]. A series of studies

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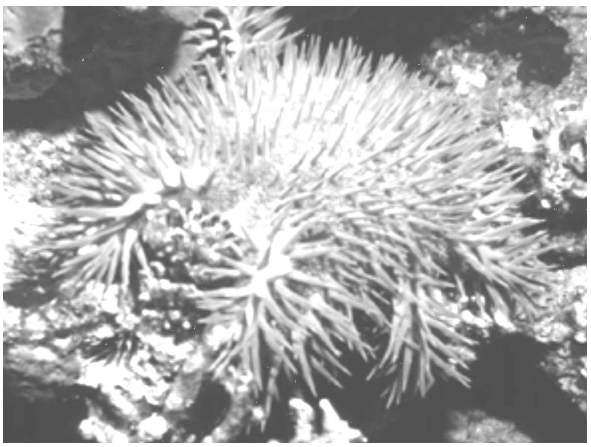
on the isolation and structure of gangliosides from starfish, *Acanthaster planci* (crown of thorn) were performed in our laboratory. Consequently, we identified ten acanthagangliosides, as shown in Fig. 1 [8,11,12]. Acanthagangliosides are classified into three types, on the basis of their oligosaccharide moiety, specially, AG-1, AG-2 (major) and AG-3. To determine the physiological functions of these gangliosides, rapid, simple, and highly sensitive assay methods are required. In this report, we describe the preparation of MAb against AG-2 and a simple determination method involving TLC immunostaining and AG-2 distribution.

Materials and Methods

Materials and chemicals

AG-2 (290.1 mg) was purified from whole bodies of *A. planci* as described previously [9]. Oligosaccharides of acanthagangliosides were prepared by enzymatic hydrolysis with endoglycoceramidase (EGCase, TAKARA, Japan) [7]. L-α-di-palmytoyl phosphatidylcholine (DPPC, Avanti), DL-α-dipalmitoylphosphatidic acid (PA, Koch-Light Laboratory), and *Salmonella minnesota* R595 lipopolysaccharides (LPS, List Biological Laboratory, USA) were obtained commercially. All other chemicals were



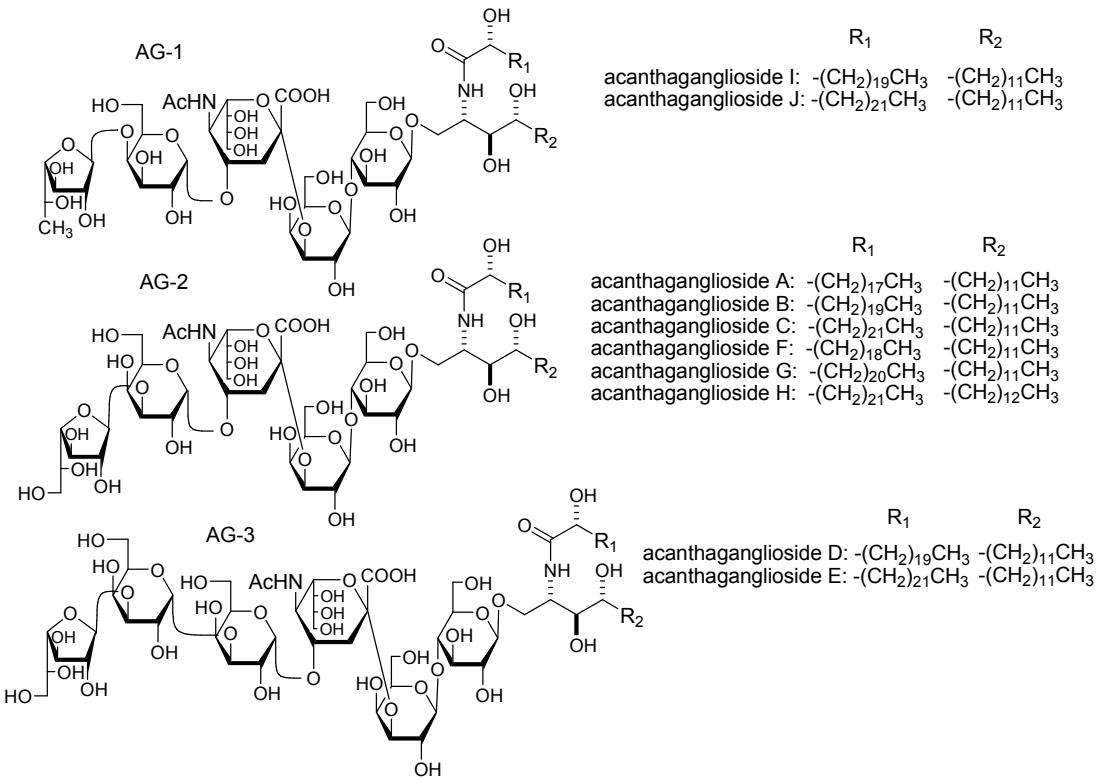


Fig. 1. *Acanthaster planci* and structures of acanthagangliosides.

standard commercial products of analytical grade.

Preparation of liposomal AG-2 antigen

Liposomes containing AG-2 for immunization were prepared according to a previous method reported by Dancey *et al.* [2]. DPPC (3.7 mg), cholesterol (2.0 mg), PA (325 mg), AG-2 (750 μg), and LPS (100 μg) were each dissolved in 1 mL of $CHCl_3$/MeOH (2:1, v/v), and mixed in a conical flask. After evaporation of the solvent using a rotary evaporator *in vacuo*, 0.15 ml of PBS was added, and the mixture incubated at 60 ˚C. The lipid film was dispersed by vigorous vortexing.

Immunization and hybridization

BALB/c male mice were injected intraperitoneally three times with liposomal AG-2 (0.15 ml; 75 μg AG-2 per injection) every 2 weeks. On the third day, after the final immunization, splenocytes were isolated and fused with a HAT-sensitive mouse myeloma cell line, P3-X63-Ag8-U1, using the polyethylene glycol (PEG) method. Hybridomas producing MAb specific for AG-2 were cloned by the limited dilution. Established hybridomas were cultured in eRDF medium (Kyokuto Seiyaku, Japan) with RD-1 supplement (Kyokuto Seiyaku, Japan).

Purification of MAbs

Cultured medium containing IgM (8D4) was filtered with a glass fiber filter (0.45 μm, Millipore, USA), followed by a cellulose acetate filter (0.22 μm, Millipore, U.S.A). The filtrate was passed though a magnesium pyrophosphate column (Kanto Chemicals, Japan), and washed with 10 mM sodium phosphate buffer (NaPB, pH 7.0). Absorbed IgM was eluted with a gradient of 10 mM to 0.5 M NaPB, then 0.5 M NaPB. The eluate was dialyzed against H_2O, and finally lyophilized to obtain pure IgM.

Reactivity of MAb to AG-2

The reactivity of MAbs to AG-2 was determined with ELISA. AG-2 (25 μg) was dissolved in EtOH (1 ml), and applied to a 96-well immuno plate (NUNC. Roskilde, Denmark) at a volume of 30 μl/well. After evaporation, PBS containing 5% skimmed milk (S-PBS) was added, and incubated for 1 hr at 37˚C. The plate was washed three times with PBS containing 0.05% Tween 20 (T-PBS) and reacted with 50 μL of testing MAb for 1hr. The plate was washed three times with T-PBS, and incubated with the secondary horseradish peroxidase-conjugated goat anti-mouse IgM antibody (Organon Teknika, U SA, 1:1000 dilution) for 1 h at 37˚C. Wells were re-washed three times as above, followed by the addition of 100 μl of substrate solution [0.1 M citrate phosphate buffer (pH 4.0) containing 0.003% H_2O_2 and 0.3 mg/ml ABTS (WAKO, Tokyo, Japan)]. After incubation for 20 min, the plate was read at 405 nm (MODEL 450 MICROPLATE READER BIO-RAD). The cross-reactivities (CR%) of related gangliosides and other compounds were determined according to Weiler's equation: CR (%) = (μg/ml of AG-2 yielding A/A0=50%) /(μg/ml of compound under investigation yielding A/A0=50 %) x 100

TLC immunostaining

AG-2 was applied to a TLC plate (Polygram Sil G, Marchery-Nagel), and developed with $CHCl_3$/MeOH/2.5N*aq*.NH_3 (6:4:0.7). The developed TLC plate was dried and incubated in a solution of S-PBS containing 1% PVP (Sigma) at 25˚C for 2 h. Following a further three washes with T-PBS, the plate was incubated in a solution of MAb (1.4 μg/ml) diluted with T-PBS containing 1% PVP at 25˚C for 24 h. Next, the plate was re-washed with T-PBS, and incubated with the secondary horseradish peroxidase-conjugated goat anti-mouse IgM antibody (diluted 1:1000) in 0.2% gelatin-PBS containing 1% PVP at 25˚C for 24 h. After washing another three times with T-PBS followed by PBS, the TLC plate was detected with PBS containing 0.03% H_2O_2 and 1% 4-chloro-1-naphthol at 25˚C for 30 min.

Extraction of AG-2 from the dissected organs

Seven major organs [pyloric caeca (PC), stomach (ST), skin (SK), abactinal spines (SP), gonads (testis (TE) or ovaries (OV)), arm (AM including an interradial longitudinal section), and tube foot (TF)] were dissected carefully from fresh materials. Organs were lyophilized and extracted with 10x ethanol. The ethanol extract was evaporated *in vacuo* to obtain ethanol ext. (50.8 mg, 1.43 g of PC; 49.3 mg, 0.88 g of ST; 79.3 mg, 2.49 g of SK; 66.7 mg, 1.66 g of SP; 77.9 mg, 1,94 g of TE; 58.4 mg, 1.64 g of AM; 15.6 mg, 0.35 g of TF). Each ethanol ext. (9 μg) was analyzed by TLC and TLC immunostaining. The TLC plate (Silicagel 60 F_{254}, Merck) was developed with $CHCl_3$/MeOH/

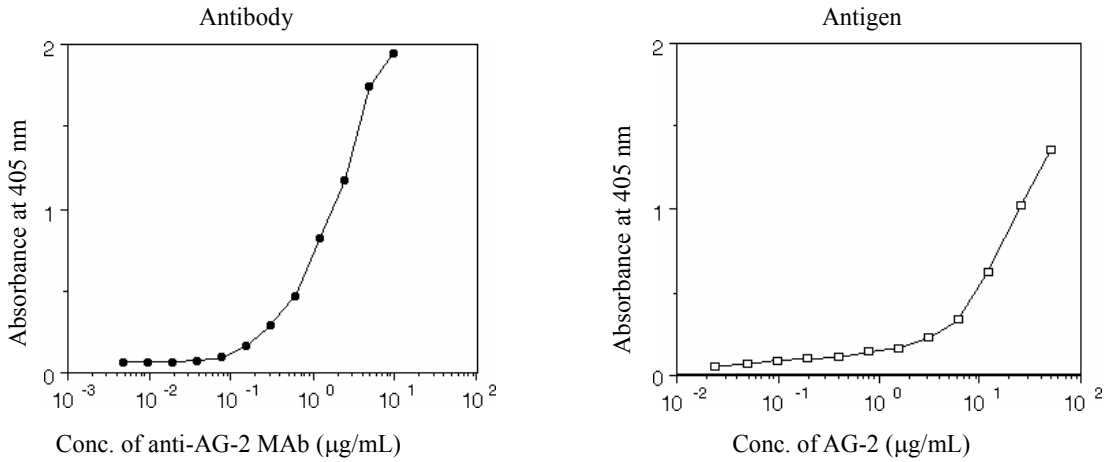


Fig. 2. Reactivity of IgM-type MAb against AG-2. The left figure shows the reactivity of various concentrations of anti-AG-2 MAb (8D4) against AG-2 and right figure depicts the reactivity of various concentrations of AG-2 against anti-AG-2 MAb in direct ELISA experiments.

2.5N*aq*.NH_3 (6:4:0.7), and detected using resorcinol-HCl and 5% H_2SO_4-MeOH.

Quantitative analysis of AG-2 using the NIH image system

The area of each spot was determined using the NIH Image 1.55 (National Institutes of Health, USA) program on a Macintosh computer, after reading in data with a scanner.

Results and Discussion

Production and characterization of MAb against AG-2

The hyperimmunized BALB/c male mice used to derive the cell clone described in this study yielded splenocytes, which were fused with P3-X63-Ag8 myeloma cells using a routine procedure. The hybridoma-producing MAb reactive for AG-2 obtained was classified as IgM with κ light chains. The reactivity of IgM-type MAb, designated 8D4, was tested at various antibody concentrations, and presented as a dilution curve (Fig. 2). Antibody and antigen concentrations of 1.6 μg/ml and 25 μg/ml, respectively, were optimized from the OD values at 405 nm of 0.8.

Purification of anti-AG-2 MAb

Fig. 3 shows the elution profile of IgM on a magnesium pyrophosphate column, and SDS-PAGE analysis of the IgM protein at each purification step is depicted in Fig. 4. Using washing solution (10 mM sodium phosphate buffer), a low concentration of IgM contaminated with a lower molecular protein was eluted. Pure IgM protein was eluted with a gradient of 10 mM to 0.5 M sodium phosphate buffer, and the heavy chain appeared at around 66 kDa, and the light chain was observed at approximately 24 kDa (lane 4 and 5 in Fig. 4). Purified IgM was employed in further experiments.

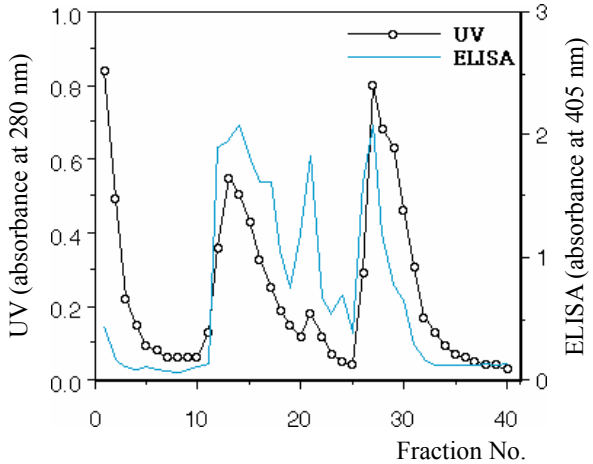


Fig. 3. Elution profile of anti-AG-2 MAb using a magnesium pyrophosphate column. Fr.1-Fr.10 (washing): eluted with 10 mM NaPB, Fr.11-Fr.25 (elution 1): eluted with a gradient of 10 mM NaPB-0.5 M NaPB, Fr.26-Fr.40 (elution 2): eluted with 0.5 M NaPB, (—): ELISA activity, (-o-): absorbance at 280 nm.

Specificity and reactivity of purified anti-AG-2 MAb

Cross-reactivity is the most important factor in determining the value of an antibody. Since direct ELISA of AG-2 was used for analytical investigations of crude

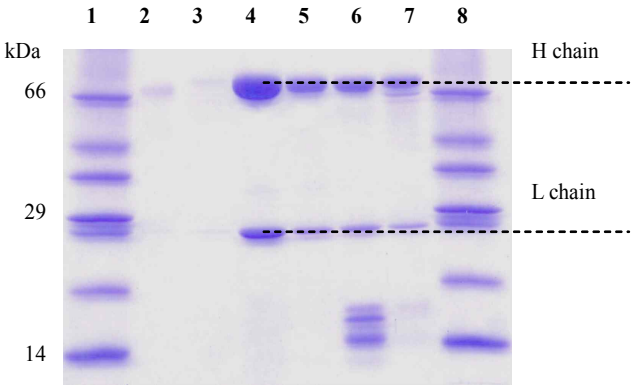


Fig. 4. SDS-PAGE analysis of IgM proteins. Lanes 1 & 8: MW marker, lane 2: standard mouse IgM, lane 3: culture medium before passing through the column (low concentration), lane 4: elution 1 (high concentration), lane 5: elution 1 (low concentration), lane 6: elution 2, lane 7: culture medium before passing through column (high concentration).

marine resource extracts, the assay specificity was yielded by determining the cross-reactivities of MAb with various related GSLs and other natural compounds. Cross-reactivities of related GSLs were determined according to the method of Weiler and Zenk [20]. Anti-AG-2 MAb displayed cross-reactivity with AG-2-Os and AG-1-Os at CR (%) of 5.03 and 0.36, respectively, but not against structurally related gangliosides, AG-1 (Fucfβ1-4Galpα1-4NeuAcα2-3-Galpβ1-4Glcpβ1-1'Cer) and AG-3 (Galfβ1-3Galpα1-4Galpα1-4NeuAcα2-3-Galpβ1-4Glcpβ1-1'Cer), as shown in Table 1. Moreover, the glycosylceramide A-1 (Glcpβ1-1'Cer) and its ceramide did not react with the antibody. These data suggest that the sugar chain is immunogenic. Thus, it is evident that MAb reacts specifically with AG-2, and not other GSLs, The newly established highly specific MAb against AG-2 may be routinely used for analytical studies on crude marine products without pretreatment.

Sensitive determination of AG-2 by TLC immunostaining

The TLC plate was immersed in anti-AG-2 MAb, followed by peroxidase-labeled secondary anti-mouse IgM MAb. Upon the addition of the substrate and H_2O_2, clear dark blue spots were observed. The detection limit for TLC immunostaining was initially determined. TLC immunostaining for different concentrations of AG-2 is depicted in Fig. 5. Lanes 1-7 represent varying amounts of AG-2 (1 μg, 0.5 μg, 0.1 μg, 50 ng, 10 ng, 5 ng, and 1 ng). Detection is possible up to 50 ng of AG-2 with this method, while AG-2 was not detected with resorcinol-HCl reagent below 1 μg.

Table 1. Cross-reactivities (%) of anti-AG-2 MAb against various compounds

Compounds	CR (%)
Gangliosides (Echinoderms)	
AG-2	100
AG-1	<0.01
Ag-3	<0.01
LMG-2	<0.01
SJG-1	<0.01
Gangliosides (Mammalian)	
GM1	<0.01
GD1a	<0.01
GD1b	<0.01
GT1b	<0.01
Cerebrosides	
A-1	<0.01
HPC-1	<0.01
Ceramides	
CER	<0.01
CJCer-1	<0.01
Oligosaccharides	
AG-2-Os	5.03
AG-1-Os	0.36
Saponins	
thornasteroside A	<0.01
QS-II	<0.01
cucumechinoside F	<0.01
Flavonoids	
daidzein	<0.01
Formononetin	<0.01
Genistin	<0.01
daidzin	<0.01
Other compounds	
Lyso-PAF	<0.01
Cholesterol	<0.01
DPPC	<0.01

LMG-2: NeuAcα2-3Galβ1-4Glcβ1-1'Cer (from starfish *Luidia maculate*), SJG-1: NeuGcα2-6Galβ1-1'Cer (from sea cucumber *Stichopus japonicus*), A-1: Galβ1-1'Cer (from starfish *A. planci*), HPC-1: Glcβ1-1'Cer (from sea cucumber *Holothuria pervicax*), CER: ceramide (from *A. planci*), CJCer: sphingosine-type ceramide (from feather star *Comanthus japonica*), AG-2-Os: oligosaccharide moiety of AG-2, AG-1-Os: oligosaccharide moiety of AG-1.

TLC immunostaining analysis of AG-2 in various organs of fresh *A. planci*

Individual EtOH extracs of organs were applied to three TLC plates, and developed with the $CHCl_3$/MeOH/NH_4OH solvent system. Fig. 6 represents immunostaining of crude EtOH extracts. The majority of AG-2 exists in the stomach of male *A. planci*, while AG-2 distributed not only in the stomach but also in

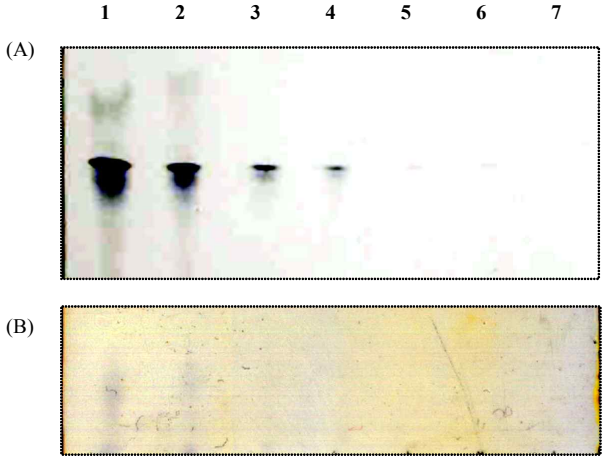


Fig. 5. TLC immunostaining of various concentrations of AG-2. A: TLC immunostaining of Polygram Sil G developed with $CHCl_3$/MeOH/2.5 N *aq*. NH_3 (6/4/0.7), lane 1: 1 μg, lane 2: 0.5 μg, lane 3: 0.1 μg, lane 4: 50 ng, lane 5: 10 ng, lane 6: 5 ng, lane 7: 1 ng of AG-2, B: TLC stained with resorcinol-HCl on Silica-gel 60 F_{254} developed under similar conditions.

the pyloric caeca of female *A. planci.* Based on immunostaining analyses, we estimated that the EtOH ext. of the stomach and pyloric caeca contain 2.7-4.1% and 3.3% AG-2 in female *A. planci*.

This is the first report on TLC immunostaining for starfish gangliosides. Our simple and rapid assay method can be routinely used for the identification of GSLs from natural resources. Moreover, the methodology may be available for GSLs assay in marine invertebrates, therefore facilitating the study of a large number of samples, as well as limited amounts of sample for identifying GLS species containing higher levels of AG-2. While it is difficult to detect small molecular compounds by Western blotting, the approach described here is particularly attractive in a wide variety of comparable situations, as evident from the distribution of GSLs in the organs of *A. planci*. Nezuo and colleagues reported that the M5 ganglioside (NeuGcα2-6Glcβ 1-1'Cer) in sea urchin exists mainly in the endoplasmic reticulum and yolk granules in unfertilized eggs. After fertilization, M5 is localized in the extracellular matrix, and transported into the cytoplasm during embryogenesis [13]. On the other hand, the starfish ganglioside AG-2 is localized in the digestive organs, and not the sex organs. It is well established that starfish reverse out their stomach and stalk prey, since the nervous system is developed and converges into the stomach. Further studies are necessary to reveal the physiological functions of the starfish gangliosides.

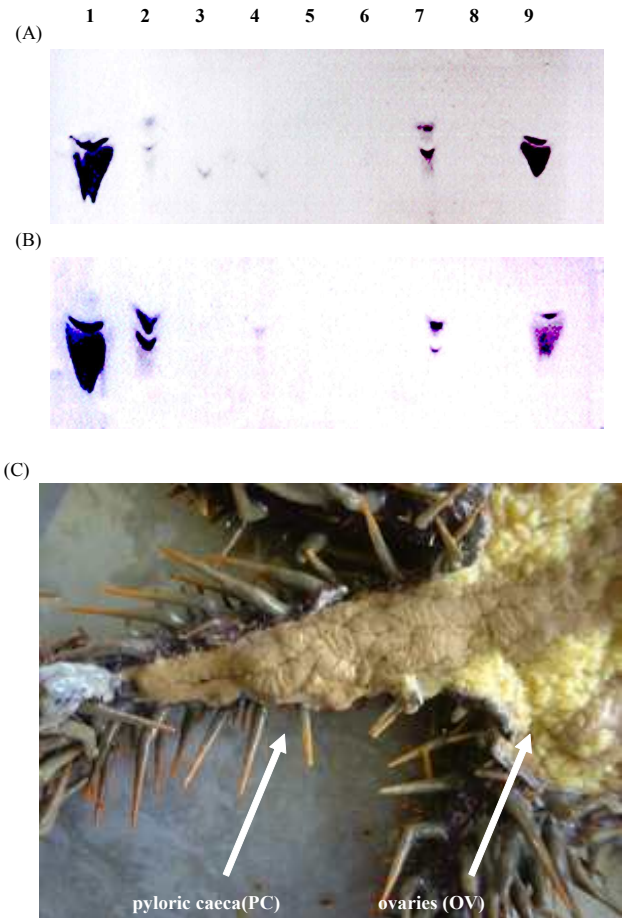


Fig. 6. TLC immunostaining of crude EtOH extracts in various organs of *A. planci*. A: TLC immunostaining of male *A.planci*, B: TLC immunostaining of female *A.planci* of Polygram Sil G developed with $CHCl_3$/MeOH/2.5 N *aq*. NH_3 (6/4/0.7), lane 1: 1 μg of AG-2, lane 2: PC, lane 3: SP, lane 4: SK, lane 5: TF, lane 6: AM, lane 7: ST, Lane 8: TE or OV, lane 9: 0.5 μg of AG-2, Fig. 6C: Internal organs of female *A. planci*

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