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MITOGENIC AND ANTINEOPLASTIC ISOAGGLUTININS FROM THE RED ALGA *SOLIERIA ROBUSTA*

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Key Word Index—*Solieria Robusta*; Solieriaceae; isoagglutinins; mitogens; glycoproteins; isolation; sugar-binding specificity; biological activity.

Abstract—Three agglutinins have been isolated from the aqueous ethanolic extract of the marine red alga *Solieria robusta* by precipitation with cold ethanol, gel filtration and ion-exchange HPLC. These new proteins, designated solnins A, B and C, were monomeric glycoproteins with a similar M_r of 23 000 from gel filtration and 29 000 from SDS-electrophoresis, and they shared such predominant amino acids as Gly, Asx and Glx. However, solnins A, B and C had different isoelectric points of 4.3, 4.2 and 4.1, respectively. From electrophoretic analyses of these agglutinins on polyacrylamide gels of various concentration, they were confirmed to be isoagglutinins. The ratio of the yields of solnins A, B and C was *ca* 1:3:1. Solnins strongly agglutinated rabbit erythrocytes, but did not agglutinate human erythrocytes and mouse FM3A tumour cells. Treatment of the rabbit erythrocytes with trypsin or pronase effected their sensitivity to hemagglutination by the agglutinins. The hemagglutinating activity of the solnins was inhibited by glycoproteins bearing *N*- glycosidic sugar chains, but not by any mono- and oligosaccharides tested. The activity was not affected by divalent cations. Solnins also showed mitogenic activity for mouse splenic lymphocytes, while they inhibited the growth in *vitro* of mouse leukaemia cells L1210 or mouse FM3A tumour cells.

INTRODUCTION

Out of 270 Puerto Rican [1], British [2–4], German [5], Japanese [6] and Spanish [7] marine algal species examined, *ca* 105 have been found to possess hemagglutinating activity. So far, however, agglutinins have been isolated and characterized only from 10 algal species [8–17]. Our systematic investigation of marine algal agglutinins has shown that algal agglutinins are of small molecular size, are monomeric and have no affinity for monosaccharides [15–18], unlike most terrestrial plant agglutinins which are composed of subunits and have an affinity for monosaccharides [19–21]. Interestingly, a major agglutinin from the red alga *Hypnea japonica* is a monomeric peptide with a very low M_r of 4200 [15]. These features of marine algal agglutinins led us to characterize other algal agglutinins.

Recently, we have isolated new agglutinins of relatively low M_rs, named solnins, with mitogenic and antineoplastic activities from the red alga *Solieria robusta* (Greville)Kylin. This paper describes the isolation and some of the properties of these agglutins.

RESULTS AND DISCUSSION

The agglutinins of *S. robusta* were extracted with 50% ethanol and recovered as a precipitate with 50–83% ethanol. This precipitate gave a single active peak on Bio gel P-60 gel filtration (Fig. 1), which was further separated into three active peaks by HPLC on a column of TSKgel DEAE-5PW (Fig. 2). The agglutinins thus obtained were homogeneous on PAGE (Fig. 3a). The three agglutinins were designated solnins A-C after the generic name of the alga. In a typical run, the yields of solnins A, B and C were 0.98, 2.55 and 0.82 mg protein, respectively, from 1 kg of the fresh material. Gel filtration on a Bio gel P-60 column revealed that each solnin had an M_r of *ca* 23 000. On SDS-PAGE, each solnin gave a single band with the same relative mobility which was estimated to have a M_r of 29 000 (Fig. 3b). They were positive to periodic acid–Schiff staining after electrophoresis. Solnins are thus small monomeric glycoproteins, similar to other agglutinins from marine algae [8, 9, 15–17], although it remains to be determined how such a monomeric form causes the agglutination of cells. It is possible

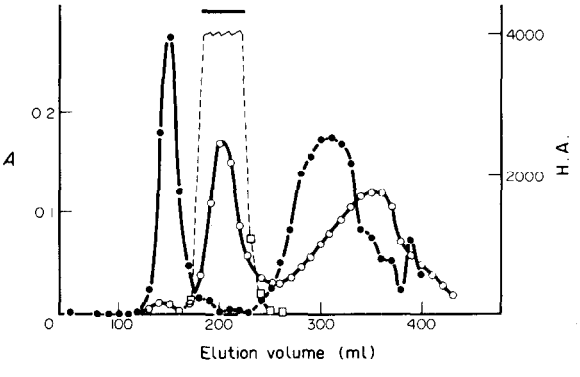

Fig. 1. Gel filtration on a Bio gel P-60 column of the crude agglutinins. ●——●, Absorption at 280 nm; ○——○, absorption at 490 nm after treatment with phenol-sulphuric acid. □——□, hemagglutinating activity. HA; hemaglutination titre. Active fractions denoted by the bar were pooled. Details are given in the Experimental.

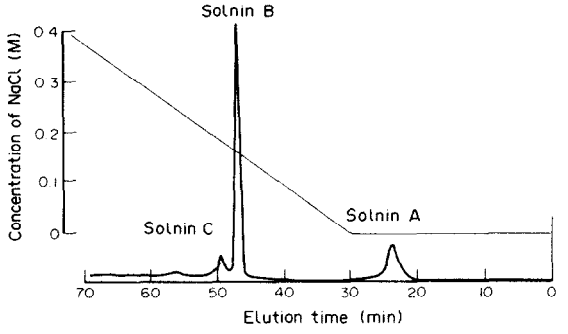

Fig. 2. HPLC on a TSKgel DEAE-5PW column of an active peak obtained by gel filtration. Details are given in the Experimental.

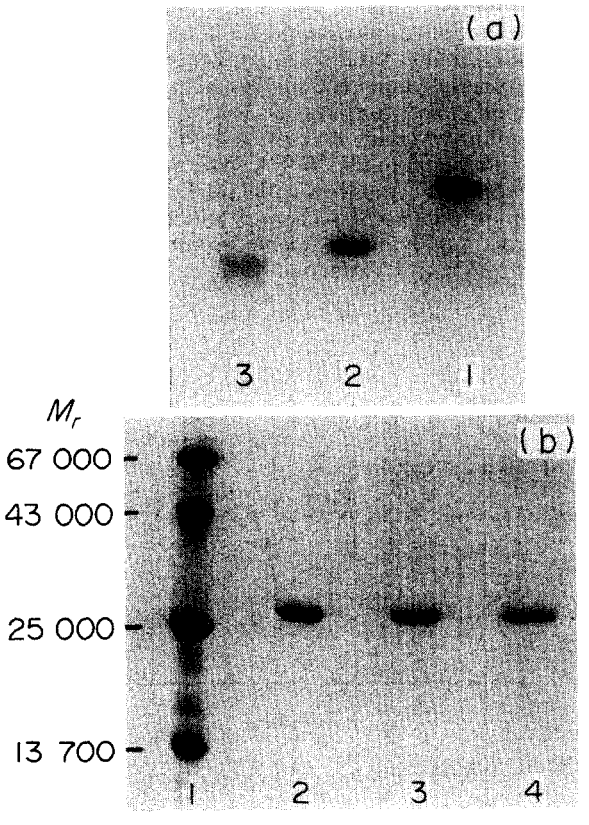

Fig. 3. (a) 7.5% PAGE of the purified agglutinins. 1; solnin A, 2; solnin B, 3; solnin C. (b) SDS-PAGE of solnins A, B and C. 1; a mixture of standard proteins, 2; solnin A, 3; solnin B and 4; solnin C.

that each solnin has at least two binding sites per molecule as seen in a subunit of a wheat germ agglutinin [21] and a *Streptomyces* sp. lectin [22], or that each agglutinin aggregates by itself on the cell surface to bring about cell agglutination. The larger value of the M_rs obtained from SDS-PAGE may be due to the acidic or glycoprotein nature of the agglutinins, because in SDS-PAGE acidic proteins or glycoproteins have a tendency to migrate more slowly and therefore to appear larger than they really are. Solnins A, B and C each gave a single band in

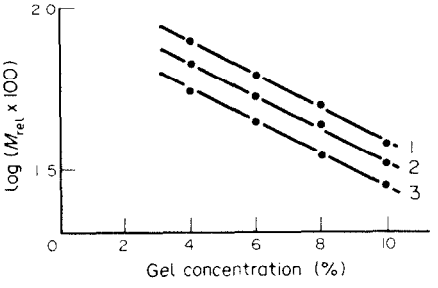

Fig. 4. Relation between relative mobilities of solnins and gel concentrations in PAGE. 1; solnin C, 2; solnin B and 3; solnin A. Details are given in the Experimental.

isoelectric focusing with pIs of 4.3, 4.2 and 4.1, respectively. When subjected to electrophoresis on polyacrylamide gels of various concentration and the logarithm of the relative mobility of each agglutinin was plotted against the corresponding gel concentration, straight parallel lines were drawn through the points with each agglutinin (Fig. 4). These results clearly indicate that solnins, A, B and C are isomeric forms. This conclusion was also supported by their similar amino acid compositions (Table 1). They shared such predominant amino acids as glycine, aspartate + asparagine, glutamate + glutamine and serine. Ornithine was also detected.

Solnins strongly agglutinated rabbit erythrocytes (Table 2). Their agglutinating activity was markedly increased with trypsin- or pronase-treated rabbit erythrocytes. The activity of solnins A, B and C were equal against untreated rabbit erythrocytes, but with enzyme-treated erythrocytes that of solnin B was the strongest. On the other hand, they showed no hemagglutination of human A, B and O erythrocytes irrespective of enzyme treatment, except for solnin A which weakly agglutinated trypsin-treated human A erythrocytes. Solnins did not

Table 1. Amino acid composition (mol %) of solnins A, B and C

Amino acid	Solnins		
	A	B	C
Asx	11.2	12.1	13.4
Thr	5.9	6.2	6.0
Ser	9.9	8.9	9.0
Glx	10.5	9.7	10.2
Pro	4.1	4.6	6.1
Gly	19.0	19.3	17.0
Ala	7.0	7.4	8.0
Val	6.3	6.8	6.4
Met	1.4	1.8	1.4
Ile	4.0	4.2	3.8
Leu	3.9	3.8	3.9
Tyr	2.4	2.8	2.2
Phe	2.3	2.3	1.9
Lys	4.6	2.2	4.2
His	0.9	0.7	0.9
Arg	3.2	3.4	2.8
Trp	2.1	3.4	1.5
Orn	1.3	0.4	1.2

Table 2. Minimum concentration of solnins A, B and C giving a positive agglutination reaction

Test cells	Minimum concentration (μg protein/ml)		
	Solnin A	Solnin B	Solnin C
Erythrocytes			
Rabbit			
Untreated	1.56	1.56	1.56
Trypsin-treated	0.024	0.012	0.200
Pronase-treated	0.003	0.0004	0.003
Human A			
Untreated	—	—	—
Trypsin-treated	25	—	—
Human B			
Untreated	—	—	—
Trypsin-treated	—	—	—
Human O			
Untreated	—	—	—
Trypsin-treated	—	—	—
Mouse FM3A tumour cells	—	—	—

Bars indicate no activity at 25 μg protein/ml.

agglutinate mouse FM3A tumour cells at the concentration of 25 μg protein/ml.

The hemagglutinating activity of solnins was not affected by heating for 30 min at 40°. Their activities were retained at a pH range between 6 and 10. but decreased below pH 5. The activities of solnins were also not affected either by treatment with EDTA or divalent cations such as Ca^{2+} and Mg^{2+}.

None of the solnins was inhibited by any of the mono- and oligosaccharides tested (Table 3). This phenomenan has seen in many other algal agglutinins [8, 10, 12, 13, 15–17]. However, they were inhibieted by glycoproteins such as fetuin, yeast mannan and ovalbumin. Transferrin and α_1-acid glycoprotein were not inhibitory, whereas desialylated transferrin was inhibitory for all the agglutinins and desialylated α_1-acid glycoprotein inhibitory only for solnin A. The glycopeptide-fraction from fetuin was also inhibitory, suggesting that the sugar moiety was responsible for inhibition. Among the inhibitory glycoproteins, yeast mannan was a best inhibitor against all the agglutinins. The *N*-glycopeptide from yeast mannan also inhibited the activities. Thus, solnins, in common with an agglutin from the red alga *Carpopeltis flabellata* [17], seem to recognize the glycoproteins bearing *N*-glycosidic sugar chains, especially of the high mannose type. The binding interaction of solnins with yeast mannan was also apparent in that the agglutinins were adsorbed on a yeast mannan-Spharose 4B column, although only a small amount of the adsorbed agglutinin was specifically eluted with 1 M D-mannose (data not shown). In this context, solnins differ from the *C. flabellata* agglutinin which are adsorbed on a yeast mannan-Sepharose 4B column and then effectively eluted with 1 M D-mannose. The solnins were not inhibited by *N*,*N*′-diacetylchitobiose and Manα1→6 (Manα1→3)Man–OMe which are the common constituent of the *N*-glycosidic sugar chains, or by mannobiose, mannotriose and mannote-

Table 3. Inhibition of hemagglutinating activity of solnins by sugars and the related compounds

Sugars and related compounds	Minimum inhibitory concentration (μg/ml)		
	Solnin A	Solnin B	Solnin C
Monosaccharides*	—	—	—
Oligosaccharides*	—	—	—
Glycoprotein			
Transferrin	—	—	—
Fetuin	125.0	500.0	250.0
α_1-acid glycoprotein	—	—	—
Asialotransferrin	500.0	1000.0	500.0
Asialofetuin	62.5	250.0	62.5
Asialo-α_1-acid glycoprotein	2000.0	—	—
Yeast mannan	7.8	125.0	7.8
Ovalbumin	1000.0	2000.0	1000.0
Glycopeptide-fraction from fetuin	+	+	+
N-Glycopeptide from yeast mannan	+	+	+
Mannosacchardide from yeast mannan			
Mannobiose	–	–	–
Mannotriose	–	–	–
Mannotetraose	–	–	–
Man α1→6 (Manα1→3)Man–OMe	—	—	—

Trypsin-treated rabbit erythrocytes were used. The minimum inhibitory concentration is that required to inhibit completely the hemagglutinating activity of a titre, 4. + and – indicate presence and absence of inhibition, respectively at a concentration of 100 mM in the case of mono- and obligosaccharides, at 2 mM in the case of Manα1→6 (Manα1→3) Man–OMe, and at 2 mg/ml in the case of glycoproteins and the other related compounds.

*Mono- and oligosaccharides tested are given in the Experimental.

traose prepared by acetolysis of the *N*-glycopeptide of the mannan. Since acetolysis cleaves only the α-(1→6)-linkage in the backbone of the yeast mannan, the mannosaccharides tested were composed of α-(1→2)- and α-(1→3)-linkages in the side chains [22]. Therefore, it is suggested that solnins recognize the moiety of α-(1→6)-linked polymannose. Thus, solnins appear to be useful reagents for elucidation of complex carbohydrate structures.

Solnins A, B and C showed mitogenic activity for splenic lymphocytes of BALB/c mice, as well as the *C. flabellata* agglutinin. Their activities were dose-dependent and the maximal activity of each agglutinin was observed at the concentration of 5–10 μg protein/ml. In this test, the activities of solnins A, B and C were equal to that of PHA(Sigma), but weaker than that of concanavalin A(Sigma). On the other hand, solnins A, B and C inhibited the growth *in vitro* of mouse leukaemia cells L1210; the IC_{50} (48-hr-old cells) values were 12, 15 and 18 μg protein/ml, respectively. These values were all lower than the IC_{50} (42 μg/ml) of concanavalin A(Sigma). Solnins A, B and C also inhibited the growth *in vitro* of mouse FM3A tumour cells at IC_{50} values of 6, 4 and 8 μg protein/ml, respectively. In these experiments, agglutination of the tumour cells was not observed. A mitogenic agglutinin from *C. flabellata* did not show the inhibitory activity for both type of the tumour cells. It is thus interesting that solnins stimulate mitosis and growth of the normal cells involved in the immune system, whereas they inhibit the growth of transformed cells.

EXPERIMENTAL

Materials. Specimens of *S. robusta* were collected on the coast of Hiroshima, Japan, in May 1985. The specimens were transferred on dry-ice to the laboratory, washed with H_2O and kept at −20° until used.

Isolation. One kg of the frozen alga was thawed and cut into small pieces. To the pieces, 900 ml 99.5% EtOH was added to give an ethanolic concn of *ca* 50%. The mixture, after addition of 200 ml 50% EtOH, was homogenized with a Ultra-Turax homogenizer and kept at 4° for 4 days with stirring. After centrifugation at 6000 rpm for 30 min, two vols of 99.5% cold EtOH (−20°) were added to the supernatant and kept at 4° overnight. The ppt was separated by centrifugation and dialysed against 0.02 M Pi buffer (pH 7) containing 0.85% NaCl (PBS). The non-dialysable fraction was concentrated by ultrafiltration and applied to a Bio gel P-60 column (2.6 × 85 cm) equilibrated with PBS. The elution was carried out with a flow rate of 40 ml/hr with PBS, and the eluate was monitored by absorption at 280 nm, sugar content and hemagglutinating activity. Active fractions were pooled, dialysed against H_2O and concentrated by ultrafiltration. The concentrate was subjected to ion-exchange HPLC on a TSKgel DEAE-5PW column (7.5 × 75 mm) equilibrated with 0.01 M Pi buffer (pH 7). The elution was performed at a flow rate of 0.5 ml/min, first with the starting buffer for 30 min, then with a linear gradient between 0 and 0.5 M NaCl in the buffer for 50 min, followed by 0.5 M NaCl in the buffer for 10 min. The eluate was monitored by absorption at 280 nm and hemagglutinating activity. Active peaks were pooled and dialysed against distilled water.

Affinity chromatography of S. robusta *agglutinins on a yeast mannan-Sepharose 4B column.* From 1 kg of another frozen specimen of *S. robusta*, a 50–83% EtOH ppt. was prepared in the same way as mentioned above. The ppt. was dissolved in PBS, dialysed against PBS and subjected to affinity chromatography on a yeast mannan-Sepharose 4B column (1 × 10 cm) equilibrated with PBS. The column was washed with PBS until the washings showed no absorption at 280 nm, and eluted with 1 M NaCl followed by 1 M D-mannose in PBS. The eluate was monitored by absorption at 280 nm and hemagglutinating activity. The affinity gel was prepared by coupling yeast mannan to CNBr-activated Sepharose 4B according to the Pharmacia mannual as follows. The CNBr-activated Sepharose 4B (15 g) (Pharmacia) was swollen in 200 ml 1 mM HCl and thoroughly washed with the dil. acid. The gel was slightly washed with 0.5 M NaCl–0.1 M $NaHCO_3$ (pH 8.3) and suspended in 150 ml of the same solvent. To the gel suspension, 400 mg of yeast mannan (Nakarai Chemicals, Japan) dissolved in 150 ml 0.5 M NaCl–0.1 M $NaHCO_3$ (pH 8.3) was added and gently stirred at 4° overnight. The mixture was filtered on a glass filter and the gel kept in 100 ml 1 M ethanolamine at room temp. for 2 hr. After filtration, the gel was successively washed with 0.5 M NaCl–0.05 M glycine–HCl buffer (pH 3), 0.5 M NaCl–0.1 M $NaHCO_3$(pH 8.3) and PBS. The affinity gel thus obtained contained 2.2 mg of yeast mannan coupled to 1 ml of the gel.

The M_r*s of the solnins* were determined by gel filtration on a Bio gel P-60 column (2.6 × 85 cm) with 0.02 M Pi buffer (pH 7) containing 0.3 M NaCl. Ovalbumin (M_r 43 000), chymotrypsinogen A(M_r 25 000) and ribonuclease A(M_r 13 700) were used as standard proteins. Blue dextran was used for estimation of the void vol.

Electrophoresis. Conventional PAGE was performed on a 7.5% polyacrylamide slab gel using Tris–HCl buffer (pH 9.4) according to the method of Davis [23]. SDS-PAGE was carried out on a 15% polyacrylamide slab gel by the method of Laemmli [24]. The preparation was dissolved in 0.01 M Tris–HCl buffer (pH 6.8) containing 2% SDS, 1 mM EDTA and 5% 2-mercaptoethanol, and boiled for 5 min. Bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), chymotrypsinogen A(M_r 25 000) and ribonuclease A (M_r 13 700) were used as standard proteins. Isoelectric focusing on a 5% polyacrylamide disc gel was carried out at 200 V for 5 hr. Ampholine pH 3.5–10/4–6 (1:4, v/v) was used as carrier ampholite at a concn of 2%. After each electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 for proteins and periodic acid–Schiff reagent for carbohydrates by the method of ref. [25]. In the case of isoelectric focusing, one gel was cut into 5 mm-slices and each slice was used for pH measurment after extraction with 1 ml of H_2O. To elucidate the nature of the isoagglutinins, the purified agglutinins were electrophoresed on polyacrylamide slab gels with gel concn of 4, 6, 8 and 10% using Tris–HCl buffer (pH 9.4), respectively. After elctrophoresis, the logarithm of relative mobility of each agglutinin was plotted against the corresponding gel concentration, according to the method of ref. [26].

Amino acid composition was determined on a Hitachi 835 amino acid analyser after hydrolysis of the sample in an evacuated tube with 6 M HCl for 24 hr at 110°. Tryptophan was determined by the thioglycol–HCl method [27].

Protein content was measured by the metod of ref. [28] using bovine serum albumin as a standard, while sugar content was measured by the method of ref. [29] using D-galactose as a standard.

Agglutinating activity was determined as described previously [18]. The cells tested were erythrocytes of rabbit, human A, B and O groups, and mouse FM3A tumour cells. Untreated and trypsin-treated erythrocyte suspensions were prepared as described previously [18]. The treatment of rabbit erythrocytes with pronase were carried out as follows; 10 mg of pronase were added to 10 ml of 2% rabbit erythrocyte suspension and the mixture incubated at 37° for 1 hr. The pronase-treated eryth-

rocytes were washed × 3 and prepared as 2% cell suspension in saline.

Hemagglutination-inhibition test was carried out with the trypsin-treated rabbit erythrocytes as described previously [18]. The sugars and glycoproteins used were; D-Glc, D-Man, D-Gal, D-GlcNAc, D-GalNAc, C-Fuc, L-Rha, D-GlcA, D-GalA, D-Man-6-P, NeuNAc, maltose, lactose, *N,N'*-diacetylchitobiose, transferrin (human), fetuin (calf), α_1-acid glycoprotein (human), ovalbumin and yeast mannan (*Saccharomyces cerevisiae*). Asialotransferrin, asialofetuin and asialo-α_1-acid glycoprotein were prepared by dialysis after hydrolysis of the parent sialoglycoproteins with 0.01 M HCl for 1 hr at 80°, respectively. The glycopeptide-fraction from fetuin was prepared according to the method of Spiro [30], as described previously [18]. The *N*-glycopeptide from yeast mannan (*S. cerevisiae*) was prepared by β-elimination followed by gel filtration according to the method of ref. [31], as described previously [18]. Mannobiose, mannotriose and mannotetraose were obtained by gel filtration of the acetolysis-products of the *N*-glycosidic glycopeptide, according to the method of ref. [22]. These glycopeptides and mannosaccharides were qualitatively examined at the concn of 2 mg/ml for inhibitory activity. The synthetic Manα1→6 (Manα1→3)Man-OMe, a kind gift from Dr T. Ogawa (The Institute of Physical and Chemical Research, Japan) was also qualitatively examined at 2 mM.

Assay method for mitogenic activity. Spleen cells were obtained from BALB/c mice in the same manner as described previously [17]. The cells were washed × 3 and resuspended in RPMI 1640 medium containing 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal calf serum to give 1×10^6 cells/ml. 200 μl of the cell suspension were placed into each well of a 96-well flat-bottled culture plate and 10 μl of a known amount of the agglutinins were added. The cultures were set up in triplicate and incubated at 37° in a 5% CO_2 incubator. After 44 hr, 10 μl of RPMI 1640 medium containing 0.5 μCi of [Me-^{3}H] thymidine was added and the cells were cultured 18 hr longer. The cells were harvested using an automatic cell harvester (Lobomash LM101, LaboScience) and dried at 50° for 5 hr. The radioactivity was determined with a scintillation counter.

Effect of solnins on the growth in vitro *of tumour cells.* Mouse FM3A tumour cells (clone 28) were cultured in suspension under 5% CO_2 in ES medium containing 2% fetal calf serum at 37°. One ml of the cell suspension (1.5×10^5 cells) containing a known amount of the agglutinin was placed into each well of a 24-well flat-bottled culture plate. The cultures were set up in triplicate and incubated at 37° in a CO_2 incubator. At 1, 2 and 3 days after the culture, the numbers of the viable cells were determined by staining with Trypan Blue. Mouse leukaemia cells L1210 were cultured in suspension under 5% CO_2 in RPMI 1640 medium containing 10% fetal calf serum at 37°. 200 μl of the cell suspension (2×10^4 cells) containing a known amount of the agglutinin was placed into each well of a 24-well-bottled culture plate. The cultures and determination of the viable cells were carried out as in the case of FM3A tumour cells.

Effect of heat, pH and divalent cations on hemagglutinating activity of solnins were determined as described previously [16].

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