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PURIFICATION AND PRELIMINARY CHARACTERIZATION OF LECTINS FROM THREE COLOR STRAINS OF THE RED MARINE ALGA, *KAPPAPHYCUS ALVAREZII* (DOTY) DOTY EX SILVA

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SUMMARY

The three lectins, named KAA-1, KAA-2 and KAA-3 after the specific name of alga, were isolated from each color strains of the red marine alga *Kappaphycus alvarezii* by combination of gel filtration and ion exchange chromatography from the 80% cold ethanol precipitates. The highest yield of KAAs was 42.8 $\mu g.g^{-1}$ fresh tissue for the red strain, next was 31.8 $\mu g.g^{-1}$ fresh tissue for the green strains, and the lowest was 30.2 $\mu g.g^{-1}$ fresh tissue for brown strains. The lectins had a molecular mass of 28000 ± 500 Da on SDS-PAGE in both non-reducing and reducing condition, respectively. Biochemical studies revealed that KAA-1 and KAA-2 are monomeric proteins without a carbohydrate moiety, and had almost identical 20 N-terminal amino acid sequence to each other, and only differed in the isoelectric point, indicating that they are isolectins to each other. The hemagglutination activities of KAAs were inhibited strongly by glycoproteins bearing high mannose type N-glycan as porcine thyroglobulin and yeast mannan so far as tested. The activities of KAAs were not affected by either the presence of EDTA or addition of divalent cations, was stable in a wide pH range from 3 to 10, and was not changed by incubation at 50°C for 30 min.

Keywords: *Carbohydrate-binding specificity, hemagglutinins, Kappaphycus alvarezii, lectins, N-terminal amino acid sequence, three color strains*

INTRODUCTION

Lectins are carbohydrate-binding proteins other than immunoglobulins, and are a highly diverse group of proteins found in most organisms, ranging from viruses and bacteria to plants and animals (Gabius, 1997; Sahron, Lis, 2003). The occurrence of hemagglutinins in extracts of marine algae was first described by Boyd *et al.* (1966). Characterization studies reveal that many of algal lectins, especially from red algae, have common characteristics of low-molecular sizes, monomeric forms, having no affinity for monosaccharides, exhibiting binding specificity for complex oligosaccharides or glycoproteins, thermostable and divalent cation-independent hemagglutination (Hori *et al.*, 1990; Rogers, Hori, 1993). Some lectins found in algae exhibit significant activity against human immunodeficiency virus (HIV) and other enveloped viruses, which makes them particularly promising targets for the development as novel antiviral drugs (De Clercq, 2005; Reeves, Piefer, 2005). Thus, algal lectins are interesting targets for basic researches and applications (Ziołkowska, Wlodawer, 2006).

The hemagglutination activity of several *Eucheuma* species was first found in the extracts by Chiles and Bird (1989) and Bird *et al.* (1993), and some of lectins of this genus were isolated with high yields including those from *E. sera, E. amakusaensis* and *E. cottonii* (Kawakubo *et al.*, 1997; 1999). Lectin ESA-2 (*E. serra*) showed various biological activities such as mitogenic activity for mouse and human lymphocytes (Kawakubo *et al.*, 1997), in vitro growth inhibition of tumor cells (Sugawara *et al.*, 2001) and antibacterial activity (Liao *et al.*, 2003). Furthermore, ESA-2 lectin remarkably suppressed colonic carcinogenesis in mice when administered orally and growth inhibition in vitro of 35 human cancer cell lines (Hori *et al.*, 2007).

Here, we describe isolation and partial characterization of lectins from three color strains of the red alga, *Kappaphycus alvarezii* cultivated in Vietnam.

MATERIALS AND METHODS

Materials

The red alga, three color strains (brown, red and green) of *Kappaphycus alvarezii* (Doty) Doty ex Silva was collected at Cam Ranh Bay, Khanhhoa Province, Vietnam, in October, 2005. After collection, the material was cleaned to remove epiphytes, washed with distilled water and used to extract protein. Human and animal red blood cells were obtained from Institute of Vaccine- Nhatrang, Vietnam and Laboratory of Marine Bioresources Chemistry, Hiroshima University, Japan. Other reagents were of analytical grade.

Purification of hemagglutinin

The fresh alga (500 g) was cut into small pieces, homogenized in a blender (1 min) with 1 volume 60% cold ethanol to attain a final concentration of 20% and kept at 4°C for 18 h with occasionally stirring. After filtration through a cheese cloth, the filtrate was centrifuged at 3000 rpm for 10 min. The supernatant was collected and examined for hemagglutination activity. To supernatant, cold absolute ethanol (−20°C) was added to attain a final concentration of 80 % and the mixture was kept at 4°C overnight. The precipitate was collected by centrifugation at 6000 rpm for 20 min and thoroughly dialyzed against 50 mM phosphate buffer containing 0.15 M NaCl (pH 7.0). The non-dialyzable fraction was applied to a Superdex R 75 HR 10/30 column equilibrated with the above buffer. Gel filtration was performed at a flow rate of 1.0 $mL.min^{-1}$ with the same buffer and the eluate monitored for absorption at 280 nm and for hemagglutination activity with trypsin- treated rabbit. The active fractions were pooled, concentrated by ultrafiltration, and dialyzed against 20 mM Tris-HCl buffer (pH 8). The concentrate was applied to ion exchange chromatography on a TSK gel DEAE-5PW column (7.5 × 75 mm) equilibrated with 20 mM Tris-HCl buffer (pH 8). The elution was performed at a flow rate of 0.4 $ml.min^{-1}$, first with the same buffer for 3 min, then with a linear gradient between 0 and 0.16 M NaCl in the buffer for 22 min, and finally with 1.0 M NaCl in the buffer for 7.0 min. The eluate was monitored for absorption at 280 nm and for hemagglutination activity with trypsin-treated rabbit erythrocytes. Active fractions were pooled and dialyzed against distilled water, separately.

Protein contents

Protein contents were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Absorbance at 280 nm was used to estimate protein content in chromatographic fractions. Sugar contents were determined by the phenol sulfuric acid method with D-glucose as a standard.

Molecular weight determination

Molecular weights of purified lectins were determined by both gel filtration on a Superdex 75 HR column (10 × 300 mm) and SDS-PAGE (Laemmli, 1970) using a 10 % polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R250. Isoelectric points were determined conventionally with isoelectric focusing on a 7% polyacrylamide gel at 460 V for 20 h using 2% Ampholine (pH 4.0 - 6.5) as carrier ampholite.

N-terminal amino acid sequence determination

The N-terminal amino acid sequence was carried out using the lectins purified by reverse-phase HPLC on an YMC PROTEIN-RP column, and were determined by Procise HT protein sequencing system (AB Applied Biosystems).

Hemagglutination and hapten-inhibition tests

Hemagglutination activity was determined with 2% (v/v) suspension of trypsin-treated rabbit erythrocytes (Hori *et al.*, 1986). Hemagglutination-inhibition tests were performed with trypsin-treated rabbit erythrocytes according to the method of Hori *et al.* (1986). Inhibition was observed macroscopically and inhibition activity was expressed as the lowest concentration (mM or μg/ml) of sugar or glycoprotein, respectively, at which a complete inhibition of hemagglutination was achieved. The following sugars and glycoproteins were tested. As monosaccharides, disaccharides, polysaccharides, D-glucose, D-mannose, D-galactose, L-rhamnose, L-fucose, D-xylose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N- acetyl-D-mannosamine, N-acetylneuraminic acid, lactose and fucoidan were purchased from Nakarai Chemical Co. As glycoproteins, transferrin, fetuin, asialofetuin, porcine thyroglobulin, bovine submaxillary mucin, porcine stomach mucin, bovine thyroglobulin, yeast mannan were from Sigma Chemical Co. Asialo-transferrin, asialo- bovine

thyroglobulin, asialo- poricin thyroglobulin and asialo-bovine submaxillary mucin were prepared by hydrolysis of the parent sialoglycoproteins with 0.05 M HCl for 1h at 80°C, followed by dialysis against saline overnight, respectively.

Effect of temperature, pH and metal ions on hemagglutination activity

To determine the effect of temperature, pH and metal ions on hemagglutination activity, trypsin-treated rabbit erythrocytes were used. Each 1ml aliquot of purified lectin solution was incubated at various temperatures from 30 ~ 100°C for 30 min, then rapidly cooled on ice and determined for hemagglutination activity. The effect of pH was determined as follows. Each 1 ml aliquot of the purified lectin was dialyzed at 4°C overnight against 0.05 M buffers of various pH values from 3 to 10 and then dialysed against saline to eliminate the pH effect. The non-dialysable fractions were measured for hemagglutination activity, respectively. The following buffers were used: acetate buffer for pH 3, 4 and 5, phosphate buffer for pH 6 and 7, Tris-HCl buffer for pH 8 and carbonate buffer for pH 9 and 10. To determine the effect of metal ions, a 1 ml aliquot of the purified lectin was dialyzed against 100 ml of 50 mM EDTA in 0.02 M phosphate buffer (pH 7) at 4°C overnight and then the hemagglutination activity of the non-dialysates was measured in saline. In order to evaluate the capacity to restore hemagglutination, the fraction was added an equal volume of 20 mM $CaCl_2$ or $MgCl_2$ in saline. After kept at room temperature for 2 h, the solution was measured for hemagglutination activity.

RESULTS AND DISCUSSION

Hemagglutination activity of the extracts

Aqueous ethanol extracts from three color strains of the red alga *K. alvarezii* agglutinated trypsin and papain-treated sheep and rabbit red blood cells but no agglutination was observed against human erythrocytes (blood groups A, B, and O), even when the cells were treated with trypsin or papain (Table 1).

Table 1. Hemagglutination activity of extracts from three color strains of *K. alvarezii*. The hemagglutination activity was expressed as a titer which is the reciprocal of the highest two-fold dilution exhibiting positive agglutination ($H.U.ml^{-1}$).

Strains	Rabbit			Sheep			Chicken			Human A			Human B			Human O		
	N	T	P	N	T	P	N	T	P	N	T	P	N	T	P	N	T	P
Brown	8	256	512	-	128	256	-	-	-	-	-	-	-	-	-	-	-	-
Red	8	256	512	-	128	256	-	-	-	-	-	-	-	-	-	-	-	-
Green	4	128	128	-	64	128	-	-	-	-	-	-	-	-	-	-	-	-

N. Native erythrocytes; T. Trypsin-treated erythrocytes; P. Papain-treated erythrocytes; -. No detected.

Isolation of lectins from three color strains of *Kappaphycus alvarezii*

The lectins from three color strains of *K. alvarezii* were efficiently extracted with aqueous ethanol and recovered as precipitates by 80% cold ethanol. The precipitates gave a single active peak on Superdex 75 gel filtration in figures 1 (A for brown, B for red and C for green strain, respectively). The active peak coincided with a major protein peak, indicating that the proteins in the precipitate consisted exclusively of lectin molecules, and was designated KAA. This was further separated into three active peaks by ion exchange chromatography on TSK gel DEAE -5PW in figures 2 (A, B and C, respectively). The three peaks of lectins from each color strain of *K. alvarezii* exhibited strong hemagglutination activities and gave a single band with the same mobility as that of KAA on SDS-PAGE (Figure 3). Therefore, they were designated KAA-1, KAA-2 and KAA-3 in the order of elution. The results of purification are summarized in table 2.

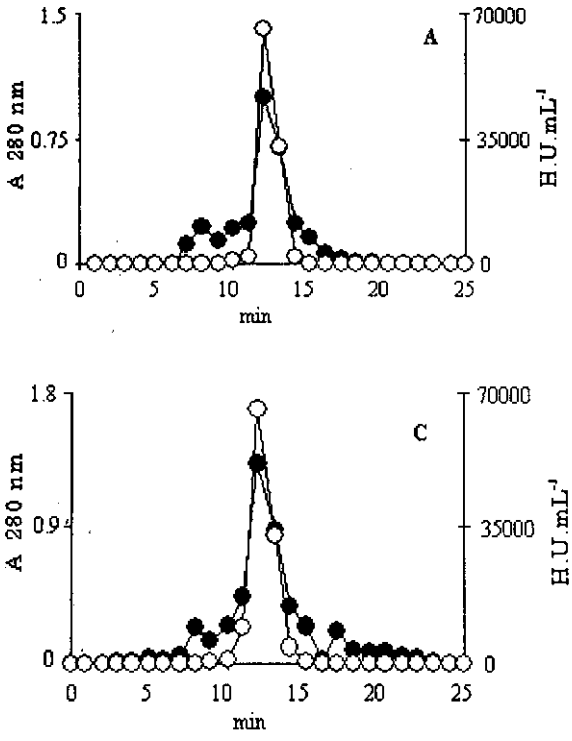

Figure 1. Gel filtration on a Superdex R 75 HR column obtained from 80% ethanol precipitates of aqueous ethanol extract, (**A**) brown, (**B**) red and (**C**) green strain, respectively. The eluate monitored for absorption at 280 nm (●—●) and for hemagglutination activity (○—○).

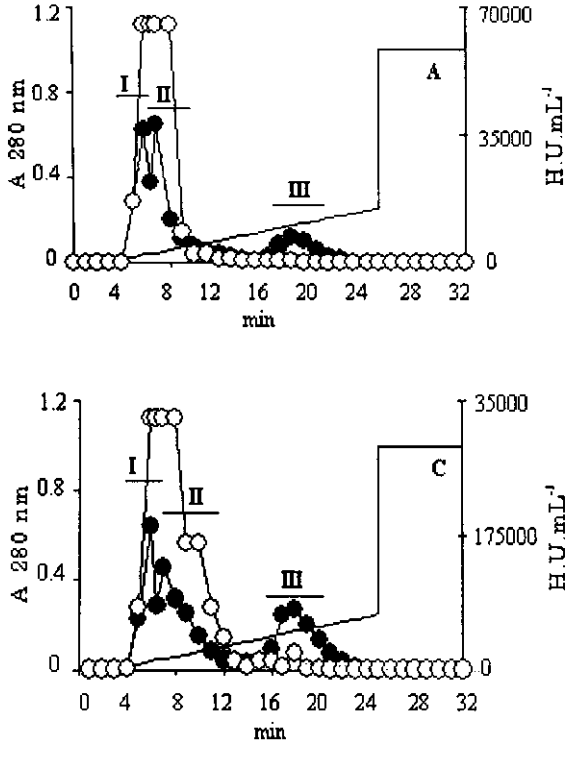

Figure 2. Ion exchange chryomatography on a TSK – GEL DEAE 5PW column of the active peak in figures 1, (A) brown, (B) red and (C) green strain, respectively. The eluate monitored for absorption at 280 nm (●—●) and for hemagglutination activity (○—○). Gradient of NaCl (–).

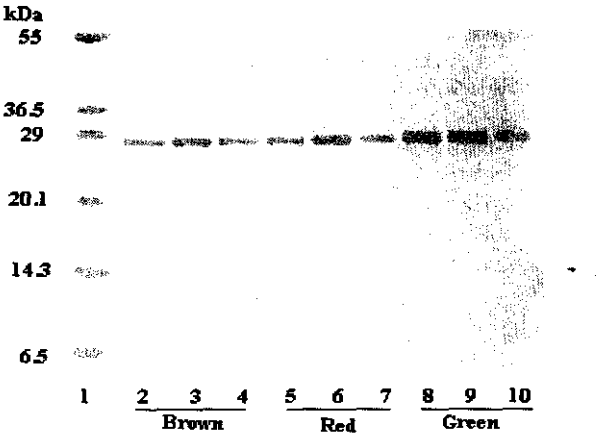

Figure 3. SDS-PAGE of the purified lectins from three color strains of *K. alvarezii*; Line 1. Marker of reference proteins; Lines 2, 3 and 4 for KAA-1, KAA-2 and KAA-3 of brown strain; Lines 5, 6 and 7 for KAA-1, KAA-2 and KAA-3 of red strain; Lines 8, 9 and 10 for KAA-1, KAA-2 and KAA-3 of green strain, respectively.

Table 2. Summary of purification of *Kappaphycus alvarezii* lectins from three color strains. Fresh alga (500 g) was used as starting material.

Kappaphycus alvarezii	Protein (mg)	H.U. ml^{-1}	MAC[a] ($\mu g.ml^{-1}$)	Total activity[b] H.U. ml^{-1}	Specific activity[c] (H.U.mg^{-1})	Yield (%)	Purification Factor
Brown strain							
20% ethanol extract	345.8	1024	0.33	1047878	3030	100.0	1.0
80% ethanol precipitation	75.6	8192	0.1	756000	10000	72.0	3.3
Gel filtration Superdex 75							
KAA	33.4	8192	0.045	742200	22000	70.8	7.3
Ion-exchange Deae-5pw							
KAA-1	5.3	8192	0.024	221757	41840	21.2	13.8
KAA-2	7.8	8192	0.015	509800	65360	48.6	21.6
KAA-3	2.0	128	0.26	7690	3845	0.7	1.3
Red strain							
20% ethanol extract	349.1	1024	0.34	1026764	2941	100.0	1.0
80% ethanol precipitation	85.3	8192	0.11	775450	9090	75.5	3.1
Gel filtration Superdex 75							
KAA	33.2	8192	0.048	691670	20830	67.4	7.1
Ion-exchange Deae-5pw							
KAA-1	6.4	8192	0.045	142200	22220	13.8	7.6
KAA-2	11.4	8192	0.023	495650	43480	48.3	14.8
KAA-3	3.6	256	0.16	22500	6250	2.2	2.1
Green strain							
20% ethanol extract	358.9	1024	0.35	1025430	2857	100.0	1.0
80% ethanol precipitation	66.8	8912	0.09	742200	11100	72.4	3.9
Gel filtration Superdex 75							
KAA	21.7	8192	0.032	678125	31250	66.1	10.9
Ion-exchange Deae-5pw							
KAA-1	3.4	8192	0.029	117240	34480	11.4	12.1
KAA-2	8.0	8192	0.021	370370	46300	36.1	16.2
KAA-3	4.5	512	0.135	33300	7400	3.2	2.6

[a]Minimum agglutination capacity (minimum amount of protein that is able to agglutinate trypsin-treated rabbit erythrocytes). [b]Inverse of the highest dilution still causing agglutination of trypsin-treated rabbit erythrocytes. [c]Hemagglutination units per mg of protein.

Chemical properties of KAA-1, KAA-2 and KAA-3

The molecular weights of KAA-1, KAA-2 and KAA-3 were estimated to be 25 000 by gel filtration on a Superdex 75 HR column and 28 000 ± 500 Da on SDS-PAGE in both non-reducing and reducing conditions, respectively. No carbohydrate was detected in lectins KAA-1 and KAA-2 of three color strains. These results implied that all the lectins were monomeric proteins. KAA-1 and KAA-2 gave a single band of pI 5.05 and 5.10 on isoelectric focusing, respectively. Thus, they are isolectins exhibiting only slight differences in isoelectric point.

Effect of temperature, pH and metal ions on hemagglutination activity

KAA-1, KAA-2 and KAA-3, the hemagglutination activities of all them were not affected by either the presence of EDTA or addition of divalent cations, thus indicating that these lectins are not metalloproteins. The activities were stable in the wide pH range from 3 to 10, and were not changed by incubation at 50°C for 30 min, whereas they gradually decreased as the incubation temperature exceeded 50°C (data not shown).

N- terminal amino acid sequences

The N-terminal amino acid sequences of these lectins up to the 20th residue were found to be identical not only to each other (Table 3), but also to those of *E. serra* lectins (ESA-1 and ESA-2). Only a replacement of Gln 6 with Lys 6 was observed for EAA-2 and EAA-3 (Kawakubo *et al.*, 1999).

Table 3. N-terminal amino acid sequences of lectins from *K. alvarezii* (KAA-1 and KAA-2), *E. amakusaensis* (EAA-1, EAA-2 and EAA-3), and *E. serra* (ESA-1 and ESA-2). - Indicates different amino acid residue.

KAA-1	GRYTVQNQWGGSSAPWNDAG...
KAA-2	GRYTVQNQWGGSSAPWNDAG...
EAA-1	GRYTVQNQWGGSSAPWNDAG...
EAA-2	GRYTVKNQWGGSSAPWNDAG...
EAA-3	GRYTVKNQWGGSSAPWNDAG...
ESA-1	GRYTVQNQWGGSSAPWNDAG...
ESA-2	GRYTVQNQWGGSSAPWNDAG...

Carbohydrate-binding specificity

The hemagglutination activity of KAA-1, KAA-2 and KAA-3 was not inhibited by any of the monosaccharides, disaccharedes and polysaccharides, but inhibited by a number of the glycoproteins bearing type O- and complex or high mannose N-glycan so far as tested (Table 4), and there was no differences in carbohydrate- inhibition tests of lectins from the three color strains. Among the inhibitory glycoproteins investigated, those bearing type high-mannose N-glycan were the strongest inhibitors such as porcine thyroglobulin (MIC = 0.97 μg. ml^{-1}), yeast mannan (MIC = 1.95 μg. ml^{-1}) and bovine thyroglobulin (MIC = 15.6 μg. ml^{-1}). Fetuin, which is an N-linked glycoprotein of the complex type with units of the disaccharide Galβ4GlcNAc (N-acetyllactosamine) substituted with sialic acid, was also inhibitory, removal of sialic acid from the parent glycoprotein by acid hydrolysis decreased the concentration of glycoprotein required to produce a similar inhibition by 2-fold, suggesting that the sugar moiety was responsible for inhibition. Bovine submaxillary mucin, which has N-acetylneuraminyl group as a terminal residue linked to the non-reducing terminal N-acetyl-D-galactosaminyl residue(s) of O-glycans, was also inhibitory. On the other hand, porcine stomach mucin with glycans rich in nonreducing terminal N-acetyl-D-galactosamine residues as well as fucose and galactose as internal residues, did not show any inhibitory activity even at concentration of 2 mg/ml.

The strong inhibition by glycoproteins bearing high-mannose type N-glycan, with respect to mannose-binding lectins, has been attributed to their high content of clustered N-glycosyl-linked mannoslyloligosaccharides, and is a feature that has been reported for lectins from genus *Eucheuma* (Kawakubo *et al.*, 1997, 1999) and other algal lectins such as cyanovirin-N (CV-N) (Boyd *et al.*, 1997), scytovirin (SVN) (Bokesch *et al.*, 2003), *Microcystis viridis* lectin (MVL) (Bewly *et al.*, 2004), griffithsin (GRFT) (Mori *et al.*, 2005), *Eucheuma sera* (ESA-2) (Hori *et al.*, 2007), and *Oscillatoria agardhii* (OAA) (Sato *et al.*, 2007). The HIV-inhibiting lectins from algae commonly showed the high mannose-binding nature that was critical for their antiviral activities (Botos, Wlodawer, 2006), and that suggested that KAA-1, KAA-2 and KAA-3 may contribute a new natural reagent source for the HIV-inhibiting activities.

Table 4. Hemagglutination-inhibition test of lectins from three color strains of ***K. alvarezii*** **with sugars and glycoproteins. Hemagglutination-inhibition test was carried out as described in materials and methods. Each value indicates the lowest concentration of sugar (mM) and glycoprotein (μg/ml) at which a complete inhibition of hemagglutination (titer, 4) was achieved.**

	Kappaphycus alvarezii								
	Brown strain			**Red strain**			**Green strain**		
Sugar & glycoprotein	KAA-1	KAA-2	KAA-3	KAA-1	KAA-2	KAA-3	KAA-1	KAA-2	KAA-3
Mono-sugar	-[a]	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-
Fucoidan	-	-	-	-	-	-	-	-	-
Transferrin	-	-	-	-	-	-	-	-	-
Asialo-transferrin	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
Fetuin	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6
Asialo-fetuin	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8
PTG	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97
Asialo-PTG	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97
BSM	31.2	31.2	31.2	31.2	31.2	31.2	31.2	31.2	31.2
Asialo-BSM	31.2	31.2	31.2	31.2	31.2	31.2	31.2	31.2	31.2
PSM	-	-	-	-	-	-	-	-	-
Yeast mannan	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95
BTG	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6
Asialo-BTG	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8

[a] Indicate no inhibition at the concentration of 100 mM for monosaccharide and 2000 μg/ml for glycoprotein. The data are presented as the minimum concentration required for inhibition of 4 hemagglutination units of the agglutinins (mM for monosaccharides and μg /ml for glycoprotein). PTG: Porcine thyroglobulin; BSM: Bovine submaxillary mucin; PSM: porcine stomach mucin; BTG: bovine thyroglobulin.

CONCLUSION

The three color strains of the red alga, *Kappaphycus alvarezii*, although there were the differences of morphologies and pigmentations, but lectin content between them was not significant differences, ranged from 30.2 to 42.8 μg. g^{-1} fresh alga, indicating that this alga will be good material for high lectin yield. The hemagglutination activity of lectins was stable in a wide range of pH and temperature. Carbohydrate-binding specificity between lectins was not the difference, and almost inhibited by glycoproteins bearing high mannose type N-glycan, suggesting that they may contribute a new natural reagent source for the HIV-inhibiting activities. Further studies are now in progress in that direction.

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TINH CHẾ VÀ MÔ TẢ SƠ BỘ ĐẶC TÍNH CỦA NHỮNG LECTIN TỪ BA DÒNG MÀU CỦA TẢO ĐỎ, *KAPPAPHYCUS ALVAREZII* (DOTY) DOTY EX SILVA

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TÓM TẮT

Ba lectin, được đặt tên KAA-1, KAA-2 và KAA-3 theo sau tên của tảo, được cô lập từ mỗi dòng màu của tảo đỏ *Kappaphycus alvarezii* bằng cách kết hợp sắc ký gel và sắc ký trao đổi ion từ các kết tủa ethanol 80%. Hàm lượng của các KAA cao nhất đạt được như sau: 42.8 µg/g tảo tươi cho dòng đỏ, tiếp đến 31.8 µg/g tảo tươi cho dòng xanh, và thấp nhất 30.2 µg/g tảo tươi cho dòng nâu. Tất cả các lectin đều có khối lượng phân tử là 28000 ± 500 Da trên SDS-PAGE trong cả hai điều kiện không khử và khử. Nghiên cứu hóa sinh cho thấy rằng các lectin KAA-1 và KAA-2 là những protein monome mà không chứa phần carbohydrate, và hầu như chúng đều có trình tự của 20 amino acid N-terminal giống nhau, chỉ khác nhau trong điểm đẳng điện, chỉ ra rằng chúng là những đồng phân lectin. Hoạt tính ngưng kết máu của các KAA bị ức chế mạnh bởi các glycoprotein mang dạng N-glycan high-mannose như porcine thyroglobulin và yeast mannan. Hoạt tính của các KAA không bị ảnh hưởng bởi sự có mặt của EDTA hoặc thêm cation hóa trị hai, bền trong một phạm vi pH từ 3 đến 10, và không bị thay đổi hoạt tính khi được đun nóng ở 50°C trong 30 phút.

Từ khóa: *Ba dòng màu, đặc tính liên kết carbohydrate, hemagglutinin, Kappaphycus alvarezii, lectin, trình tự amino acid N-terminal*

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