NOTE

Structure and anticancer activity of a new lectin from the cultivated red alga, *Kappaphycus striatus*

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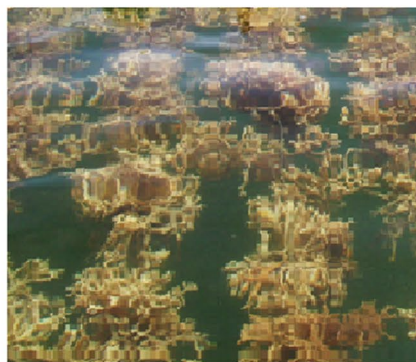
Abstract

The red alga *Kappaphycus striatus* is economically important food species and extensively cultivated throughout most tropical parts of the world as a source of carrageenan. In this note, the primary structure of a new lectin KSL from this alga was elucidated by the rapid amplification method of complementary DNA (cDNA) ends, which consists of 267 amino acid residues distributed in four tandem-repeated domains of about 67 amino acids and sharing 43% of identity. The calculated molecular mass from the deduced sequence was consistent with that of natural KSL (27,826 Da) determined by electron spray ionization–mass spectrometry. The primary structure of KSL showed high similarity to those of the high mannose N-glycan specific lectins from marine red algae, ESA-2 from *Eucheuma serra*, EDA-2 from *Eucheuma denticulatum*, KSA-2 from *Kappaphycus striatum*, KAAs from *Kappaphycus alvarezii* and SfLs from *Solieria filiformis*, and from microorganisms, BOA from *Burkholderia oklahomensis*, MBHA from *Myxococcus xanthus*, OAA from *Oscillatoria agardhii* and PFL from *Pseudomonas fluorescens*. Furthermore, KSL showed anticancer effects against five carcinoma cell lines, HT29, Hela, MCF-7, SK-LU-1 and AGS, in a dose-dependent manner with the IC_{50} values of 0.80–1.94 μM, whereas its inhibition activities on cancer cells were not detected in the presence of yeast mannan, an inhibitor against lectin KSL. The cultivated red alga *K. striatus* could also be a good source of functional lectin(s) for application as anticancer agents.

Graphic abstract

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GRYTVQNQWGGSSAPWNDAGLWILGSRGNQNVMAIDVNSSDGGANLNGTMTYSGEGPIGFKGARRGES (1-68)
NVYDVENQWGGSSAPWHAGGQFVIGSRSGQGVLAVNITSSDGGKTLTGTMTYEREGPIGFKGTQSGG- (69-135)
DTYNVENQWGGSSAPWNKAGIWALGDRSGQAMIAMDVSSSDGGKTLEGTMQYKGEGPIGFRGKLSGA- (136-202)
NNYSVENQWGGSSAPWNKAGDWLIGDRHNQNITAVKVSSDNDGKNLDGTCTYESEGAIGFKGVAS--- (203-267)
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Kappaphycus striatus
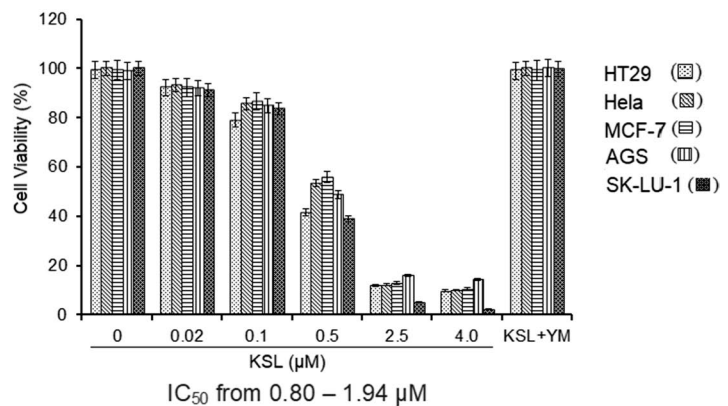
IC_{50} from 0.80 – 1.94 μM

HT29, Hela, MCF-7, AGS, SK-LU-1

Extended author information available on the last page of the article

Introduction

Lectins, carbohydrate-binding proteins, are present in various organisms ranging from viruses to humans, and play important roles as recognition molecules in cell–cell or cell–matrix interactions. Due to the ability to discriminate differences in carbohydrate structures, not only lectins are available as potential reagents in many research fields but they are promising candidates for medicinal and clinical application [1].

Marine algae are a good source of novel lectins. Algal lectins possess unique molecular structures and carbohydrate-binding specificities distinct from known lectins from other sources, which make them useful for applications [2, 3]. Recently, some the high-mannose specific lectins from the eukaryotic marine red algae attracted some attention as potential sources of new lectins with antibacterial, antiviral (HIV, SARS-CoV and influenza virus) and anticancer activities [4–11]. Thus, marine algal lectins may become a novel source of antiviral and anticancer compounds for biochemical and medicinal applications.

Cancer, the commonest cause of death, is a heterogeneous disease characterized by a variety of glycosylation and genetic alterations that induce growth and survival [12]. Currently, chemotherapy is the most commonly used method to treat cancer. However, chemotherapeutic techniques are limited by their high cellular toxicity, as well as the occurrence of side effects [13]. Therefore, recent selection of biological agents to treat certain types of different tumors has attracted some attention for applications [14].

In Vietnam, the red alga *Kappaphycus striatus* has been cultivated commercially since 2005, due to transport of algal seeds from Bohol, Philippines [15]. In this note, we cloned the cDNA encoding a new lectin KSL and elucidated the primary structure as anticancer activity on five carcinoma cell lines, which may provide valuable information for application as functional food lectin(s).

Results and discussion

A new lectin KSL from red alga *K. striatus* was purified by a combination of extraction with aqueous ethanol, ethanol precipitation, gel filtration and ion-exchange chromatography with the yield of 25.1 mg from 1 kg of the frozen algal powder (Table 1). The relative molecular mass of the lectin was estimated to be about 28,000 Da in both non-reducing and reducing SDS-PAGE conditions, indicating that lectin exists in monomeric form (Fig. 1a). By electron spray ionization-mass spectrometry, the molecular mass of KSL was determined to be m/z: 27,826 Da (Fig. 1b), which agrees well with the apparent molecular mass of 28 kDa

Table 1 The summary of purification of KSL from *K. striatus* fraction

	Protein (mg)	Total activity ($\times 10^3$ HU[a])	Specific activity ($\times 10^3$ HU/mg)	Yield (%)
Extraction	1266.5	1767	1.4	100.0
Ethanol precipitation	422.1	1275	3.0	72.2
Gel filtration	156.1	965	6.2	54.6
Ion-exchange	25.1	305	12.2	17.2

[a]Hemagglutination units

Fig. 1 a SDS-PAGE of the purified KSL lectin from the red alga *K. striatus*. Lane 1, a mixture of reference proteins (New England BioLabs, Ipswich, Massachusetts, USA); lane 2 and 3, active fractions obtained from ion-exchange chromatography in absence and presence of 2-mercaptoethanol, respectively. **b** ESI–MS deconvoluted mass spectra of KSL
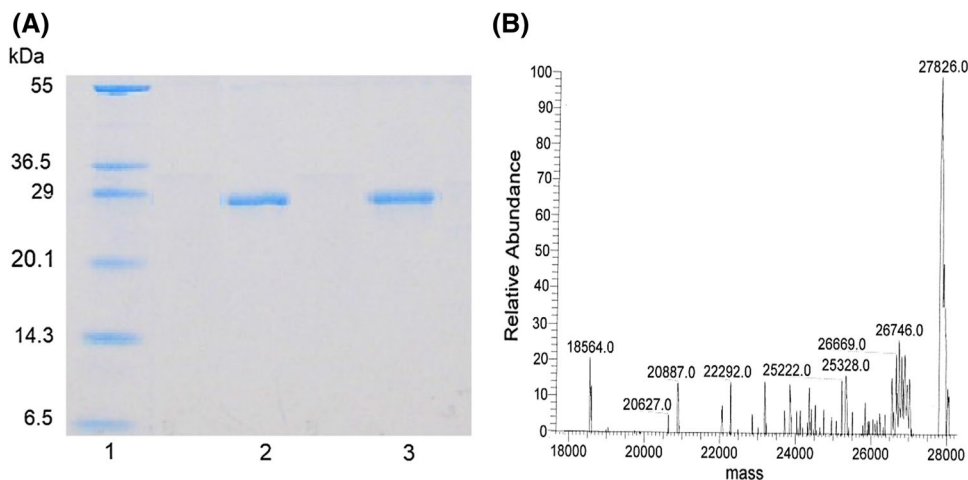
observed in SDS-PAGE. The hemagglutination activities of KSL were not inhibited by any of the monosaccharides but strongly inhibited by yeast mannan with a minimal inhibitory concentration of 1.9 µg/ml, which has high-mannose type N-glycans in the molecules, indicating that the lectin is specific for high-mannose N-glycans.

The cDNA of KSL consisted of 1066 bp containing 76 bp of 5′ untranslated region (5′UTR), 804 bp of an open reading frame (ORF) and 186 bp of 3′ untranslated region (3′UTR) (Fig. 2). The ORF coded a polypeptide of 268 amino acids including an initiating methionine. The primary structure of KSL has four tandem-repeated domains, consisting of

67 amino acids each and the domains share 43% of identity each other (Fig. 3). KSL contains one cysteine residue at position 253 (Fig. 2), which did not appear to be involved in disulfide bond formation. The calculated molecular mass of the deduced amino acid sequence in KSL cDNA was 27,825.2 Da, which was consistent with that of KSL (27,826 Da) determined by ESI–MS (Fig. 1b). Small variations around these molecular masses were observed, suggesting the presence of adduct formations. The 20 N-terminal amino acid sequence of KSL, which had been determined by Edman degradation found in the deduced amino acid sequence of KSL cDNA.

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ATAGCTGAGTCAAGTTACACCAACCTCTAAAACGCCACATCAGACTCTCTAATCTCTTCAT      61
TATCTTCAATTTGTGATGGGACGTTACACAGTTCAGAACCAATGGGGGGGATCCTCTGCC     121
              M  G  R  Y  T  V  Q  N  Q  W  G  G  S  S  A       15
CCCTGGAATGATGCCGGCTTGTGGATCCTTGGCAGTCGTGGCAACCAGAATGTGATGGCT     181
 P  W  N  D  A  G  L  W  I  L  G  S  R  G  N  Q  N  V  M  A       35
ATTGATGTTAACTCCAGCGATGGAGGTGCCAACCTGAACGGTACGATGACCTACTCCGGT     241
 I  D  V  N  S  S  D  G  G  A  N  L  N  G  T  M  T  Y  S  G       55
GAAGGCCCGATTGGATTCAAGGGGGCACGCCGTGGTGAGTCAAATGTTTACGATGTAGAG     301
 E  G  P  I  G  F  K  G  A  R  R  G  E  S  N  V  Y  D  V  E       75
AACCAGTGGGGAGGATCTTCCGCCCCCTGGCACGCTGGAGGCCAATTCGTTATCGGTTCC     361
 N  Q  W  G  G  S  S  A  P  W  H  A  G  G  Q  F  V  I  G  S       95
AGGTCTGGACAGGGAGTGCTTGCCGTGAACATTACTTCTTCAGATGGCGGAAAGACATTG     421
 R  S  G  Q  G  V  L  A  V  N  I  T  S  S  D  G  G  K  T  L      115
ACTGGCACCATGACCTATGAGAGAGAAGGTCCAATTGGATTCAAGGGTACTCAGTCTGGC     481
 T  G  T  M  T  Y  E  R  E  G  P  I  G  F  K  G  T  Q  S  G      135
GGTGACACTTACAACGTAGAGAACCAGTGGGGTGGGTCCTCGGCGCCGTGGAACAAGGCA     541
 G  D  T  Y  N  V  E  N  Q  W  G  G  S  S  A  P  W  N  K  A      155
GGTATTTGGGCGCTCGGGGATCGCAGTGGGCAAGCAATGATTGCTATGGATGTTTCGTCC     601
 G  I  W  A  L  G  D  R  S  G  Q  A  M  I  A  M  D  V  S  S      175
TCGGATGGTGGAAAAACTCTAGAGGGGACGATGCAGTACAAAGGAGAGGGACCGATTGGA     661
 S  D  G  G  K  T  L  E  G  T  M  Q  Y  K  G  E  G  P  I  G      195
TTCCGTGGCAAGCTGAGCGGTGCCAACAACTACAGTGTCGAGAACCAGTGGGGCGGTTCC     721
 F  R  G  K  L  S  G  A  N  N  Y  S  V  E  N  Q  W  G  G  S      215
TCTGCTCCTTGGAACAAGGCTGGAGACTGGTTGATTGGAGACCGCCACAATCAAAACATA     781
 S  A  P  W  N  K  A  G  D  W  L  I  G  D  R  H  N  Q  N  I      235
ACCGCAGTCAAGGTGTCATCTGACAATGATGGAAAGAACCTCGACGGCACATGCACGTAC     841
 T  A  V  K  V  S  S  D  N  D  G  K  N  L  D  G  T  C  T  Y      255
GAGAGCGAGGGTGCCATTGGTTTCAAGGGAGTCGCTTCCTAATCTAAAGAGCGTTTGAGC     901
 E  S  E  G  A  I  G  F  K  G  V  A  S  *                        268
GCAAGGATCAGAACACTCCTTGGCGGATAGATCCGAACATTTAGTAGGTGTTTCTTGTTT     961
CCCTCATGATCAGTAGCCAATCATCTCTCAAAGTTCACAACAGAGTAAAAACGTGATCAC    1021
CCTCTAAGAGTTTGCAACTTAGGAAGGAGGCTTTGACAATTTCGT                   1066
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Fig. 2 Nucleotide and deduced amino acid sequences of KSL. Underlines indicate 20 N-terminal amino acid sequence determined by Edman degradation method. The italicized first amino acid residue represents an initiation methionine. The stop codon TAA is shown as an asterisk. The italicized and non-italicized numbers represent the positions of nucleotides and amino acids, respectively

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GRYTVQNQWGGSSAPWNDAGLWILGSRGNQNVMAIDVNSSDGGANLNGTMTYSGEGPIGFKGARRGES  (1-68)
NVYDVENQWGGSSAPWHAGGQFVIGSRSGQGVLAVNITSSDGGKTLTGTMTYEREGPIGFKGTQSGG-  (69-135)
DTYNVENQWGGSSAPWNKAGIWALGDRSGQAMIAMDVSSSDGGKTLEGTMQYKGEGPIGFRGKLSGA-  (136-202)
NNYSVENQWGGSSAPWNKAGDWLIGDRHNQNITAVKVSSDNDGKNLDGTCTYESEGAIGFKGVAS---  (203-267)
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Fig. 3 Comparison of four tandem-repeated domains of KSL. Identical amino acids are shaded

KSL shared high similarity with sequences of the high-mannose binding lectins from the eukaryotic marine red algae and the prokaryotic microorganisms found in the database (Fig. 4), including ESA-2 from *E. serra* (GenBank accession no. P84331), EDA-2 from *E. denticulatum* (Gen-Bank accession no. LC057379), KSA-2 from *K. striatum* (GenBank accession no. LC057282), KAA-1 and KAA-2 from *K. alvarezii* (GenBank accession no. LC007080 and LC007081, respectively), SfL-1 and SfL-2 from *S. filiformis* (GenBank accession no. C0HL89 and C0HL90, respectively), BOA from *B. oklahomensis* (GenBank accession no. AIO69853), MBHA from *M. xanthus* (GenBank accession no. M13831), OAA from *O. agardhii* (GenBank accession no. P84330) and PFA from *P. fluorescens* Pf0-1 (GenBank accession no. AIO69853). KSL showed 98.1%, 94.0%, 98.9%, 99.3%, 98.5%, 81.7% and 79.0% of sequence identity with ESA-2, EDA-2, KSA-2, KAA-1, KAA-2, SfL-1 and SfL-2, respectively, whereas bacterial lectins BOA, MBHA, OAA and PFA showed lower sequence identities to KSL with values of 57.0%, 59.8%, 63.6% and 62.9%, respectively. All contain four tandem-repeated sequences of about 67 amino acids, except OAA and PFA which are composed of only two tandem-repeated homologous domains. The seven amino acid residues per a single repeat domain "WGGREGP", which interact with mannopentaose of a branched oligomannosyl core of Man_8/Man_9 in BOA [16] were absolutely conserved in all the repeated domains among lectins, except only a replacement at position 259 of proline by alanine in the fourth repeat domain of KSL, which was not found out among these lectins (Fig. 4). The structural kinship suggested that these lectins might have similar high-mannose binding specificities and biological activities.

The assays revealed that KSL at doses of 0.02 μM and 0.1 μM did not reduce the viability of HT29, Hela, MCF-7, AGS and SK-LU-1 cells to a significant level compared to the controls at 0 μM of KSL, respectively ($P > 0.05$), (Fig. 5). KSL at doses of 0.5 μM, 2.5 μM and 4.0 μM showed a reduction of cell viabilities remained at 41.7% ($P = 0.019$), 11.9% ($P < 0.01$) and 9.7% ($P < 0.01$) for HT29; 53.5% ($P = 0.036$), 12.2% ($P < 0.01$) and 9.9% ($P < 0.01$) for Hela; 55.8% ($P = 0.042$), 12.8% ($P < 0.01$) and 10.5% ($P < 0.01$) for MCF-7; 48.7% ($P = 0.024$), 15.9% ($P < 0.01$) and 14.3% ($P < 0.01$) for AGS; and 39.0% ($P = 0.016$), 5.0% ($P < 0.01$) and 3.1% ($P < 0.01$) for SK-LU-1 cells, respectively, as compared to the controls at 0 μM of KSL (Fig. 5). KSL showed anticancer

effects in a dose-dependent manner with the IC_{50} values of HT29 (0.99 ± 0.12 μM), Hela (1.69 ± 0.19 μM), MCF-7 (1.94 ± 0.25 μM), AGS (1.63 ± 0.18 μM) and SK-LU-1 cells (0.80 ± 0.04 μM), depending on target cell lines. As compared to KSL, Ellipticine, a cancer cell cytotoxic agent used as positive control, exhibited inhibition effects against cancer cells with the IC_{50} values for HT29 (1.86 ± 0.08 μM), Hela (1.58 ± 0.16 μM), MCF-7 (1.91 ± 0.20 μM), AGS (1.90 ± 0.20 μM) and SK-LU-1 cells (1.70 ± 0.16 μM) (data not shown). Recently, increasing interest has been shown in marine lectins because of their pro-apoptotic, cytotoxic and antiproliferation effects over different cell lines. These lectins present high specificity for the large number of glycoprotein receptors on tumor cell membranes [1–3]. ESA lectins from red alga *E. serra* inhibited the growth of HeLa and Col201 cells (human colon adenocarcinoma) at the concentrations above 1.2 μg/ml and caused the complete death of Colo201 cells at 30 μg/ml (approximately 1.07 μM). Nevertheless, MCF-7 cells had relatively high tolerance for ESA, and the cell viability was maintained above 40% even at the high doses [17]. SfL lectins from the red alga *S. filiformis* showed the induction of the apoptotic cell death on MCF-7 cells with value of $IC_{50} = 125$ μg/ml (approximately 4.5 μM) [11]. PFL lectin from bacterium *P. fluorescens* Pf0-1 presented also a significant effect on decreasing the viability of MKN28 cells (human gastric cancer) at dose of 0.5 μM or higher by 72 h [18]. Although these lectins share repeated domain structures and high-mannose binding specificities, but they differ from each another in amino acid sequences, which may lead to subtle differences in the degree of their inhibiting activities [11, 17, 18].

The inhibition activities of KSL toward cancer cell lines were not detected in the presence of yeast mannan bearing high-mannose N-glycans (Fig. 5). Similar case has been reported for PFL lectin, its effect on MKN28 cells was inhibited in the presence of yeast mannan [18]. Sugahara et al. [17] verified that the cell viability induced by ESA lectins was decreased after the treatment of Colo201 cells with α-mannosidase, β-mannosidase and endoglycosidase H to cleave high mannose type sugar chains on cell surface. These data combine with the recent reports on the presence of the high-mannose structures on the surface of Hela and MCF-7 cells [19–21] corroborate that the lectins interacted with cancer cells through their surface mannose branches. Therefore, the anticancer activity of KSL is able to specifically bind

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KSL    GRYTVQNQWGGSSAPWNDAGLWILGSRGNQNVMAIDVNSSDGGANLNGTMTYSGEGPIGFKGARRGE    67
ESA-2  GRYTVQNQWGGSSAPWNDAGLWILGSRGNQNVMAVDVNSSDGGANLNGTMTYSGEGPIGFKGARRGE    67
EDA-2  GRYTVQNQWGGSSAPWNDAGLCILGSRGNQNVIAVDVTSSDGGANLGGTMTYSGEGPIGFKGARRGE    67
KSA-2  GRYTVQNQWGGSSAPWNDAGLWILGSRGNQNVMAIDVNSSDGGANLNGTMTYSGEGPIGFKGARRGE    67
KAA-1  GRYTVQNQWGGSSAPWNDAGLWILGSRSNQNVMAIDVNSSDGGANLNGTMTYSGEGPIGFKGARRGE    67
KAA-2  GRYTVQNQWGGSSAPWNDAGLWILGSRGNQNVMAIDVNSSDGGANLNGTMTYSGEGPIGFKGARRGD    67
SFL-1  GRYTVQNQWGGSSAPWNDAGLWLLGSRANQNVMDVSVTSSDGGATLTGTMTYSGEGPIGFKGTRRGD    67
SFL-2  GRYTVQNQWGGSSAPWNDAGVFVLGGRANQNVMAIDVSSSDGGKTLTGTMTYSGEGPIGFKGTRRGE    67
BOA    NLQHVQNQWGGSSAPWHEGGMWVLGCRSGQNVVALNIKSGDGGRTLTGTMTYVGEGPIGFRATLTQ-    75
MBHA   AAYLVQNQWGGSQATWNPGGLWLIGARDKQNVVALDIKSDDGGKTLKGTMTYNGEGPIGFRGTLSS-    66
OAA    ALYNVENQWGGSSAPWNEGGQWEIGSRSDQNVVAINVESGDDGQTLNGTMTYAGEGPIGFRATLLG-    66
PFL    SKYAVANQWGGSSAPWHPGGTWVLGARDNQNVVAIEIKSGDGGKSFTGTMTYAGEGPIGFKAQRTG-    66

KSL    SNVYDVENQWGGSSAPWHAGGQFVIGSRSGQGVLAVNITSSDGGKTLTGTMTYEREGPIGFKGTQSG   134
ESA-2  SNVYDVENQWGGSSAPWHAGGQFVIGSRSGQGVLAVNITSSDGGKTLTGTMTYEREGPIGFKGTQSG   134
EDA-2  SNVYDVENQWGGSSAPWHAGGQFVIGSRSGQGVTALSVTSSDGGKTLTGTMTYEREGPIGFKGTQSG   134
KSA-2  SNVYDVENQWGGSSAPWHAGGQFVIGSRSGQGVLAVNITSSDGGKTLTGTMTYEREGPIGFKGTQSG   134
KAA-1  SNVYDVENQWGGSSAPWHAGGQFVIGSRSGQGVLAVNITSSDGGKTLTGTMTYEREGPIGFKGTQSG   134
KAA-2  SNVYDVENQWGGSSAPWHAGGQFVIGSRSGQGVLAVNITSSDGGKTLTGTMTYEREGPIGFKGTQSG   134
SFL-1  SNNYDVENQWGGSSAPWHAGGTFVIGSRSGQGVVAVDVNSSDGGKTLTGTMTYANEGPIGFKGTQSG   134
SFL-2  SNNYEVENQWGGSSAPWHPAGTFVIGSRSGQAVVAMNVTSHDGGKTLSGHMTYENEGPIGFKGTQAE   134
BOA    SNTYAVENQWGGSSAPWHPGGTWVIGCRVNQQVVALDIESGDQGATLAGTMTYAGEGPIGFKSQQAD   142
MBHA   ANNYTVENQWGGTSAPWQPGGVWVLGARDKQNIVAVSIKSNDGGKTLTGTTTYNGEGPIGFKSEVTD   133
OAA    NNSYEVENQWGGDSAPWHSGGNWILGSRENQNVVAINVESGDDGQTLNGTMTYAGEGPIGFKGTLT-   132
PFL    QNYYNVENQWGGNDAPWHPGGKWVIGGRDNQNVIALSVTSSDGGKNLSGTNTYANEGPIGFRGQIE-   132

KSL    GDTYNVENQWGGSSAPWNKAGIWALGDRSGQAMIAMDVSSSDGGKTLEGTMQYKGEGPIGFRGKLSG   201
ESA-2  GDTYNVENQWGGSSAPWNKAGIWALGDRSGQAMIAMDVSSSDGGKTLEGTMQYKGEGPIGFRGKLSG   201
EDA-2  GDTYNVENQWGGSSAPWNKAGIWALGDRNGQAMIAMDVSSSDGGQTLEGTMQYKGEGPIGFRGKLSG   201
KSA-2  GDTYNVENQWGGSSAPWNKAGIWALGDRSGQAMIAMDVSSSDGGKTLEGTMQYKGEGPIGFRGKLSG   201
KAA-1  GDTYNVENQWGGSSAPWNKAGIWALGDRSGQAMIAMDVSSSDGGKTLEGTMQYKGEGPIGFRGKLSG   201
KAA-2  GDTYNVENQWGGSSAPWNKAGIWALGDRSGQAMIAMDVSSSDGGKTLEGTMQYKGEGPIGFRGKLSG   201
SFL-1  GDSYNVENQWGGSSAPWNKAGAWALGDRDGQGVIGVDVTSSDGGKTLTGTMQYQNEGPIGFKGTSTG   201
SFL-2  GDTYNVENQWGGSSAPWNKAGVWALGSRASQGVVKLDVSSSDGGKTLTGTMQYQNEGPIGFRGTLTG   201
BOA    GGVYAVENQWGGSSAPWHNGGVWVIGARDQ-AVVAVSIGSTDGGKTLNGNMTYAGEGPIGFKGNSVA   208
MBHA   GDTYSVENQWGGSAAPWHSGGVWVLGTRGKQNVINVDAKSNDGGKTLSGTMTYNGEGPIGFRGTLTS   200

KSL    ANNYSVENQWGGSSAPWNKAGDWLIGDRHNQNITAVKVSSDNDGKNLDGTCTYESEGAIGFKGVAS--   267
ESA-2  ANNYSVENQWGGSSAPWNAAGDWLIGDRHNQNITAVKVSSDNDGKNLDGTCTYEREGPIGFKGVATS-   268
EDA-2  ANNYAVENQWGGSSAPWNKAGDWLIGDRYNQNITAVKVSSDNDGKNLDGTCTYEREGPIGFKGVATS-   268
KSA-2  ANNYSVENQWGGSSAPWNKAGDWLIGDRHNQNITAVKVSSDNDGKNLDGTCTYEREGPIGFKGVATS-   268
KAA-1  ANNYSVENQWGGSSAPWNKAGDWLIGDRHNQNITAVKVSSDNDGKNLDGTCTYESEGPIGFKGVAS--   267
KAA-2  ANNYSVENQWGGSSAPWNKAGDWLIGDRHNQNITAVKVSSDNDGKNLDGTCTYEREGPIGFKGVATS-   268
SFL-1  GSNYKVENQWGGSSAPWNPAGNWLIGDRHNQNIVAVKVTSSDNGKTLGGTCTYEREGPIGFKGTAI--   267
SFL-2  ANNYKAENQWGGSSGAWNPAGLWLIGDRHNQNIIGVKVTSDDNGKTLEGTCTYYREGPIGFKGVAN--   267
BOA    GNNYAVENQWGGTSAPWHPGGIWLLGCRSGQNVVELYITSGDNGNTFHGSMTYSGEGPIGFRAMALPQ   276
MBHA   PDTYTVENQWGGSTAPWNPGGFWMIGARNGQNVVALNVASSDGGKTLAGTMIYNGEGPIGFRARLG--   266
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Fig. 4 Alignment of amino acid sequence of KSL and other lectin sequences. ESA-2 from *E. serra*, EDA-2 from *E. denticulatum*, KSA-2 from *K. striatum*, KAA-1 and KAA-2 from *K. alvarezii*, SfL-1 and SfL-2 from *S. filiformis*, BOA from *B. oklahomensis*, MBHA from *M. xanthus*, OAA from *O. agardhii* and PFA from *P. fluorescens* Pf0-1. The identical amino acids are indicated by shading. Residues underlined are amino acids of the carbohydrate binding sites according to Ref. [16], except position 259 of alanine in the fourth repeat domain of KSL
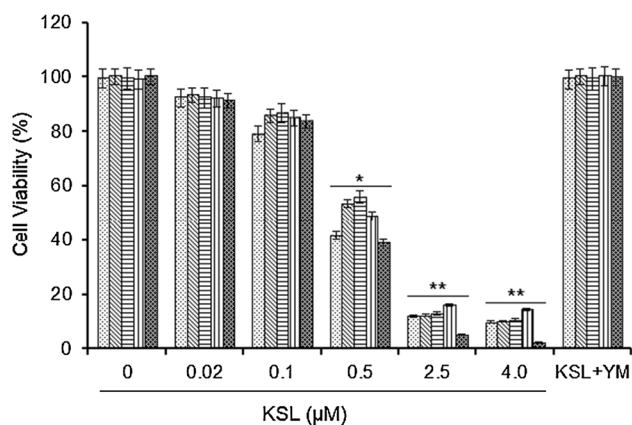
Fig. 5 Effect of KSL on the cancer cell viability. Cancer cells were treated with various doses of KSL. KSL + YM, the cells were treated with a mixture of KSL and yeast mannan (YM). The cell viability (%) was measured by sulforhodamine B dye test. HT29 cells (▨), Hela cells (▧), MCF-7 cells (▤), AGS cells (▥) and SK-LU-1 cells (▦). The mean ± SEM ($n = 3$). *$P < 0.05$ and **$P < 0.01$ for the cancer cell lines treated by KSL was compared with controls at 0 µM of KSL

between lectin and high-mannose oligosaccharides present on the surface of the cancer cells. The results suggested that KSL has the potentiality to represent broad activities against anticancer cells possessing high-mannose glycans on their surface. However, to obtain a better understanding of anticancer effect, we need studies furthermore in details of each cell line. It is important that anticancer lectin is isolated from a globally cultivated edible algal species and will become a strong tool by being supplied in bulk for application.

Materials and methods

Alga and cell lines

The red alga *K. striatus* (F. Schmitz) Doty ex Silva was collected in the farm at Ninhthuan province (109° 02′ 01″ E, 11° 35′ 23″ N), Vietnam, brought to the laboratory and kept at − 20 °C until use. A small portion of alga was stored at − 20 °C in RNAlater (Ambion, Thermo Fisher Scientific, Austin, Texas, USA) until used for the RNA extraction. The cancer cell lines, human colon carcinoma cell (HT29), human cervical carcinoma cell (Hela), human breast adenocarcinoma cell (MCF-7), human gastric adenocarcinoma cell (AGS) and human lung carcinoma cell (SK-LU-1) were supplied from Long Island University (Brooklyn, New York, USA) and University of Milan (Milan, Italy).

Purification of lectin

Frozen algae were ground to a fine powder in the presence of liquid nitrogen. The powder was extracted with 20% aqueous

EtOH (v/v) and kept at 4 °C for 18 h with occasional stirring. Insoluble algal material was removed by centrifugation at 3500g for 30 min at 4 °C. To the supernatant, cold absolute EtOH (− 20 °C) was added to attain a final concentration of about 80% and the mixture was kept at 4 °C overnight. The resulting precipitates were collected by centrifugation and thoroughly dialyzed against 50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. The non-dialyzable fraction was applied to a Sephacryl S-200HR column (GE Healthcare, Uppsala, Sweden) and eluted with the same buffer, the active fraction is 4096 hemagglutination unit/ml, concentrated by ultrafiltration, and dialyzed against 20 mM Tris–HCl buffer (pH 8.0). The concentrate was subjected to ion-exchange chromatography on a TSKgel DEAE-5PW column (Tosoh Corporation, Tokyo, Japan) equilibrated with 20 mM Tris–HCl buffer (pH 8.0). This elution was performed at a flow rate of 0.5 ml/min, first with the same buffer for 10 min, then with a linear gradient between 0 and 0.16 M NaCl in the buffer for 20 min, and finally with 1.0 M NaCl in the buffer for 10 min. The eluate was monitored at an absorbance of 280 nm for protein and for hemagglutination activity with a 2% (v/v) suspension of trypsin-treated rabbit erythrocytes. Active fractions were pooled, dialyzed against distilled H$_2$O and stored at − 20 °C until used.

Hemagglutination assay

Hemagglutination assays were carried out using a microtiter method in a 96-well microtiter V-plate. First, 25 µl amounts of serially two-fold dilutions of a test solution were prepared in saline on a microtiter V-plate. To each well, 25 µl of a 2% trypsin-treated rabbit erythrocytes [22] was added and the mixtures were gently shaken and incubated at room temperature for 2 h. Hemagglutination activity was expressed as a titer, the reciprocal of the highest twofold dilution exhibiting positive hemagglutination. The assay was carried out in triplicate for each test solution.

Hemagglutination-inhibition test

First, 25 µl each of the serially twofold dilutions of sugar (100 mM) or glycoprotein (2000 µg/ml) were prepared in saline. To each well, an equal volume of a lectin solution showing the hemagglutination titer 4 was added, and the plate was mixed gently and allowed to stand at room temperature for 1 h. Finally, 25 µl of a 2% suspension of trypsin-treated rabbit erythrocytes was added onto each well, and the plate was gently shaken and incubated for another 1 h. Inhibition activity was expressed as the lowest concentration of sugar or glycoprotein. ᴅ-Glucose, ᴅ-mannose, ᴅ-galactose, ʟ-fucose, *N*-acetyl-ᴅ-glucosamine, *N*-acetyl-ᴅ-galactosamine, *N*-acetyl-ᴅ-mannosamine, *N*-acetylneuraminic acid and yeast mannan were obtained from Nacalai Tesque

(Kyoto, Japan). Transferrin, fetuin and porcine thyroglobulin were from Sigma-Aldrich (Saint Louis, Missouri, USA).

Determination of molecular mass and N-terminal amino acid sequence of lectin

The molecular mass of purified lectin was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel [23]. The sample was heated at 100 °C for 5 min with or without 2% 2-mercaptoethanol. After SDS-PAGE, the gel was stained with Coomassie brilliant blue R-250 for proteins. Prior to the determination of molecular mass by electron spray ionization-mass spectrometry (ESI–MS) and N-terminal amino acid sequence, lectin was purified by HPLC on an YMC PROTEIN-RP column (YMC, Kyoto, Japan) which had been equilibrated with 20% acetonitrile in 0.1% trifluoroacetic acid (TFA). After injection of the sample, the column was washed with the starting solvent and then eluted with a linear gradient of acetonitrile from 20 to 70% in 0.1% TFA. The eluate was monitored for absorbance at 280 nm. The peak containing lectin was recovered, dried and dissolved in 50% acetonitrile in 0.1% TFA for determining molecular mass by ESI–MS using Finnigan LCQ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and N-terminal amino acid sequence by Procise HT protein sequencing system ABI (Thermo Fisher Scientific).

Rapid amplification of the 3′ and 5′ cDNA ends (3′ and 5′ RACEs) of KSL

Total RNA of alga *K. striatus* was extracted from the RNAlater-treated algal tissues by using the plant RNA isolation reagents (Invitrogen, Thermo Fisher Scientific, Carlsbad, California, USA). Messenger RNA (mRNA) purification from the total RNA was performed using an Oligotex-dT30 mRNA purification Kit (Takara Bio Inc., Kusatsu, Japan). First-strand cDNAs were synthesized from mRNA of alga using a GeneRacer Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instruction.

The first polymerase chain reaction (PCR) for rapid amplification of the cDNA 3′ end (3′RACE) of KSL was performed with a 10 µl reaction mixture containing 1 µl of a 10× Blend Taq buffer (Toyobo, Osaka, Japan), 2 pmol of each deoxynucleotide triphosphate (dNTP), 6 pmol of the GeneRacer_3′_Primer, 5′-GCTGTCAACGATACGCTA CGTAACG-3′, 2 pmol of KSL_common_F1 primer, 5′-AGA ACCAGTGGGGAGGATCT-3′, 0.2 µl of a tenfold diluted synthesized cDNA, and 0.25 units of Blend Taq DNA polymerase (Toyobo). The reactions were performed with a T Gradient Thermocycler (Analytik Jena, Jena, Germany) under the following conditions, denaturation at 94 °C for 5 min, followed by 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at temperatures of 60 °C for 30 s,

and extension at 72 °C for 1 min, and the final extension step at 72 °C for 5 min. The first PCR products for KSL were subcloned into pGEM-T Easy vector (Promega, Madison, Wisconsin, USA). DNA sequencing was performed using BigDye Terminator Cycle Sequencing Kit Ver. 3.1 with ABI 3130xl DNA sequencer (Applied Biosystems, Thermo Fisher Scientific).

The first PCR of 5′RACE of KSL was performed in the same way as 3′RACE, using GeneRacer_5′_Primer, 5′-CGA CTGGAGCACGAGGACACTGA-3′ and the degenerated primer KSL_5′RACE_d_R1, 5′-AYTGRTTYTCIACR TTRTAITGRTC-3′ as the primer pairs. The nested PCR was performed by the same method, except for using 1 µl of the dilution of the first PCR products as a template, using GeneRacer_5′_Nested_Primer, 5′-GGACACTGA CATGGACTGAAGGAGTA-3′ and the degenerated primer KSL_5′RACE_d_R2, 5′-ATIGGICCYTCICCYTTRTAY TGC-3′ as the primer pairs. Subcloning and DNA sequencing were performed as described above.

To verify the sequence accuracy, full-length cDNA of KSL was further amplified with high fidelity DNA polymerase KOD FX Neo (Toyobo) with primer pairs of KSL_5′ End_F, 5′-ATAGCTGAGTCAAGTTACACCAAC-3′, and KSL_3′ End_R, 5′-ACGAAATTGTCAAAGCCTCC-3′, which were designed from the 5′ and 3′ terminal sequences of KSL cDNA obtained by 5′RACE and 3′RACE. Subcloning and DNA sequencing were then performed as described above.

Homologous sequences were searched with the basic local alignment search tool (BLAST) program. Amino acid sequence comparison with homologous proteins from various organisms was performed using Clustal Omega [24].

Cell culture and viability assay

The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific) supplemented with 15% of heat-inactivated Fetal Bovine Serum (Gibco, Thermo Fisher Scientific) and 1% of Penicillin–Streptomycin-Fungizon (Sigma-Aldrich) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells (2×10^4/200 µl/well) were seeded into a 96-well flat-bottom microtiter plate (Corning Life Sciences, Tewksbury, Massachusetts, USA) in DMEM medium containing various doses of KSL dissolved in 10 mM phosphate-buffered saline (pH 7.4) and incubated for 48 h. The adherent cell cultures were fixed in situ by adding 50 µl of cold 50% trichloroacetic acid and incubated for 60 min at 4 °C. The supernatant was then discarded, and the plates were washed five times with deionized water and dried. One hundred µl of sulforhodamine B solution (SBR) (Sigma-Aldrich) (0.4% in 1% acetic acid) was added to each well and the culture was incubated for 10 min at room temperature. Unbound SRB was removed by

washing five times with 1% acetic acid. Then the plates were air dried. Bound stain was solubilized with 10 mM unbuffered Tris base, and the optical densities were read on ELISA Plate Reader (Bio-Rad) at a wavelength of 515 nm [25]. Ellipticine (Sigma-Aldrich) was used as positive control and phosphate-buffered saline instead of KSL as negative control and examined as described above. The inhibition activity of KSL on cancer cell lines was also determined in the presence of yeast mannan. KSL (4.0 μM) was pretreated with yeast mannan (2000 μg/ml) for 2 h and mixture was examined in the same way as described above. The experiments were performed in triplicate. The IC_{50} values were expressed as cytotoxic activities.

Statistical analysis

All results were confirmed by at least three independent experiments. Statistics are presented as the mean $\pm$ SEM. Statistical significance was analyzed by one-way ANOVA followed by Tukey's post-hoc test. Differences were considered significant when $P < 0.05$. The IC_{50} values were calculated by using the software GraphPad Prism, version 5.00.

Compliance with ethical standards

Conflict of interest The authors have read and approved the manuscript. We declare no conflicts of interest.

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