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Purification and Characterization of a Rhamnose-Binding Chinook Salmon Roe Lectin with Antiproliferative Activity toward Tumor Cells and Nitric Oxide-Inducing Activity toward Murine Macrophages

Clara Shui Fern Bah,[†] Evandro Fei Fang,[‡] Tzi Bun Ng,[‡] Sonya Mros,[§] Michelle McConnell,[§] and Alaa El-Din Ahmed Bekhit*[,†]

[†]Department of Food Science, University of Otago, Dunedin, 9016, New Zealand

[‡]School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China

[§]Department of Microbiology and Immunology, University of Otago, Dunedin, 9016, New Zealand

ABSTRACT: In this study, a rhamnose-binding lectin from the roe of chinook salmon (*Oncorhynchus tshawytscha*) was purified and characterized, and its biological activities were examined in several model systems. Chinook salmon roe lectin had a molecular mass of 30 kDa and agglutinated rabbit and bovine erythrocytes. The hemagglutination activity of the lectin was not affected by metal ions. The lectin was stable up to 70 °C and between pH 4 and pH 11. Chinook salmon roe lectin did not exert antifungal activity toward the fungal species tested and did not exhibit mitogenic response toward mouse splenocytes up to a concentration of 5 mg/mL. The lectin had selective antiproliferative activity toward human breast cancer MCF-7 cells and hepatoma Hep G2 cells. It also induced the production of nitric oxide from mouse peritoneal macrophages. This is the first report that demonstrates these biological activities from chinook salmon roe lectin.

KEYWORDS: Chinook salmon, roe, lectin, characterization, antiproliferative, nitric oxide

INTRODUCTION

Lectins comprise a group of proteins or glycoproteins, which are of nonimmune origin. They are found in a wide variety of species in nature including fish and are thought to play a role in innate immunity.[1] They bind reversibly to carbohydrates and agglutinate cells. Because of their biochemical properties, they have become a useful tool in several fields of biological research including immunology,[2] membrane structure and cell recognition,[3] cancer research,[4] and clinical microbiology.[5]

Lectins have been identified in the roe of several fish species. Interestingly, these fish roe lectins have been found to possess a range of different bioactivities. For example, lectin from cobia ovary was reported to have antibacterial activity toward *Escherichia coli*;[6] lectin from roach eggs exhibited mitogenic activity toward human lymphocytes;[7] loach egg lectin induced the release of cytokines from fresh murine bone marrow cells[8] and had tumor cell agglutination capability;[9] and lectin from grass carp egg induced cytokine gene expression in both murine splenocytes and cells from peritoneal exudates.[10]

Lectins have been purified from the roe of different species of salmon including chum salmon,[11,12] coho salmon,[13] and chinook salmon.[14,15] Studies using salmon roe lectins have primarily focused on investigating their antibacterial activity. Lectin from chum salmon ova reportedly induced agglutination of *Vibrio anguillarum*, a pathogenic fish bacterium.[11,12] However, agglutination cannot be equated with antibacterial activity because the bacterium continued to proliferate. Voss et al.[14] reported 90% inhibition of the same microorganism, *V. anguillarum*, by a lectin from chinook salmon ova as compared to controls. On the other hand, Yousif et al.[13] found that the lectin from coho egg salmon had neither agglutinating nor inhibiting activity toward *V. anguillarum*. While it appears that all of the salmon eggs contain lectins, the bioactivity of each particular lectin differs depending upon the species of salmon from which it originated. Other than the antibacterial activity of lectins from salmon roes, very little is known about their biological activities.

In this study, a lectin from the roe of New Zealand chinook salmon (*Oncorhynchus tshawytscha*) was purified and characterized. Furthermore, an investigation into the biological activities of chinook salmon roe lectin (CSRL) in terms of mitogenic, antiproliferative, nitric oxide (NO) induction, and antifungal activities was carried out.

MATERIALS AND METHODS

Materials and Reagents. The Bradford kit for assay of protein concentration was from Bio-Rad Laboratories (Hercules, CA). All chromatographic media and columns including CM-Sepharose, SP-Sepharose (Fast Flow), Q-Sepharose (Fast Flow), Mono S 5/50 GL, and Superdex 75 10/300 GL columns were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). All chemical reagents were purchased from Sigma-Aldrich Co (St. Louis, MO) unless otherwise stated. Animal blood (rabbit, chicken, sheep, and bovine) used in the hemagglutination assay was obtained from the Animal Welfare Facilities (Hercus Taieri Resource Unit), University of Otago. BALB/c and C57 mice were also provided by the Taieri Resource Unit, University of Otago, and handled following appropriate ethical recommendations from the University of Otago Animal Ethics Committee. Human hepatocellular carcinoma Hep G2 and human breast tumor MCF-7 cell lines were purchased from the American Type Culture Collection (United States).

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Sample Collection and Processing. Mature chinook salmon roe was obtained from Akaroa Salmon New Zealand Ltd. (Christchurch, New Zealand). The samples were vacuum packed and stored at −80 °C until analysis. Salmon roe was homogenized with an equal weight to volume ratio of distilled water, and the crude extract was centrifuged at 10000 rpm for 30 min at 4 °C. The solution was then filtered through filter paper, and the resulting supernatant, referred to as the salmon roe crude extract, was used for the purification and characterization of lectin as described below.

Purification of Lectin. Ammonium acetate buffer (1 M, pH 4.5) was added to the crude roe extract until the concentration of NH_4OAc attained 20 mM. The supernatant was then loaded on a CM-Sepharose column (2.5 cm × 10 cm), which had been pre-equilibrated with 20 mM NH_4OAc buffer (pH 4.5). The unbound fraction was eluted with the same buffer. The bound fraction with hemagglutination activity (HA) was then eluted from the column with 1 M NaCl in the same buffer. Fractions containing HA were pooled, dialyzed extensively against deionized water overnight in a chiller at 4 °C, lyophilized, dissolved in 20 mM NH_4OAc buffer, and loaded onto an SP-Sepharose column (2.5 cm × 10 cm). The column was washed with the equilibration buffer to elute the unbound fraction until OD280 reached the baseline. The adsorbed fraction was then eluted from the column with 1 M NaCl in 20 mM NH_4OAc buffer.

After exhaustive dialysis against deionized water at 4 °C, lyophilization, and then reconstitution in 20 mM NH_4OAc buffer (pH 4.5), the SP-Sepharose bound fraction was applied to a Mono S 5/5 GL column and eluted with two linear concentration gradients of NaCl (0−0.2 M and then 0.2−1 M). Fraction MS-2 eluted with the second gradient was then dialyzed and lyophilized. This fraction was subjected to final purification step using a Superdex 75 10/300 GL column using in 0.2 M NH_4HCO_3 buffer (pH 9.4) as the elution buffer. The purified lectin was obtained as one major peak (SUP 1), dialyzed extensively, freeze-dried, and stored at −80 °C for further analyses.

Protein Concentration Measurements. The concentration of soluble proteins was determined by the Bradford assay[16] using bovine serum albumin (BSA) as a standard.

Purity and Molecular Mass (MW) Determination by Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed using a 12% polyacrylamide gel as described by Laemmli and Favre[17] and Fang et al.[18]

Purity and MW Determination by Fast Protein Liquid Chromatography (FPLC)-Gel Filtration. FPLC-gel filtration, carried out using a Superdex 75 HR 10/30 column that had been calibrated with MW standards (GE Healthcare) was also employed to determine the purity and MW of the protein as described by Fang et al.[18]

Determination of N-Terminal Amino Acid Sequence. N-Terminal amino acid sequence analysis was carried out using an HP 1000A Edman degradation unit and an HP 1000 HPLC system (Hewlett-Packard). Sequence alignment was done using CluxtalX1.83, and the consensus alignments were shaded by BOXSHADE 3.21.[18]

Assay of HA. The hemagglutinating activity of CSRL was determined by measuring agglutination of rabbit, sheep, chicken, and bovine red blood cells using 2-fold serial dilutions of the lectin. A 50 μL volume of CSRL (2.5 mg/mL) and 2-fold serial dilutions in 20 mM sodium phosphate buffer containing 0.15 M NaCl (pH 7.2) were mixed with 50 μL of 2% erythrocyte suspension in round-bottom microtiter plates (96-well) as described by Lam and Ng.[10] Titer was defined as the reciprocal of the highest dilution giving visible agglutination of the rabbit erythrocytes after 1 h of incubation.

Inhibition of Hemagglutination by Sugars. The hemagglutination inhibition test was performed in a manner analogous to the hemagglutination test and as described by Yousif et al.[13] The carbohydrate-binding specificity of the purified lectin was assessed by the ability of a series of sugars (L-arabinose, D-fructose, D-fucose, D-galactose, *N*-acetyl-glucosamine, D-xylose, D-galactose, L-rhamnose, melibiose, raffinose, lactose, and maltose) to inhibit the agglutinating activity of the lectin against native rabbit erythrocytes. The lowest concentration of a specific sugar that inhibited hemagglutination was defined as the minimum inhibitory concentration (MIC).

Heat Stability Assay. As only a small amount of purified lectin was obtained, the heat stability assay was conducted using salmon roe crude extract. The heat stability of lectin in salmon roe crude extract was determined by incubating 500 μL aliquots of the crude extract (freeze-dried powder dissolved at a concentration of 2 mg/mL) at 30, 40, 50, 60, 70, 80, and 90 °C for 10, 20, 30, and 60 min. The samples were then cooled at 4 °C for 30 min and assayed for hemagglutinating activity at room temperature.[19] The agglutination activity obtained by assaying the lectin at room temperature was defined as 100% activity.[20]

Effect of pH on HA. The effect of pH on HA was determined according to the method of Suseelan et al.[21] Used were the following buffers: 50 mM acetate buffer (pH 4.0−5.0), 50 mM phosphate buffer (pH 6.0−7.0), 50 mM Tris−HCl buffer (pH 8.0−9.0), and 50 mM sodium bicarbonate buffer (pH 10.0−11.0). The agglutination value obtained with 20 mM PBS (pH 7.2) was considered to equal 100% activity.

Effects of Acid and Alkali on HA. The effect of acid and alkali on HA was determined according to the method of Ng et al.[22]

Effects of Metal Ions on HA. The effects of metallic chlorides including calcium chloride, manganese chloride, ferric chloride, magnesium chloride, and zinc chloride on the HA of CSRL were investigated as described by Akev and Can.[23] To the first of 12 wells of each series, 50 μL of a 0.01 M solution of a metallic salt (in 0.9% NaCl) was mixed with 50 μL of lectin solution. Controls were made with 50 μL of NaCl instead of the metallic salt solution. After incubation at room temperature for 1 h, 2-fold dilutions were made in 50 μL of 0.9% NaCl up to well number 12. Then, 50 μL of a 2% rabbit erythrocyte suspension was added to each well. Agglutination was estimated visually after standing for 2 h at room temperature. A second control experiment containing only the metal salt and blood cells was run concurrently to ascertain the effect of each metal on the red blood cells in the assay procedure.

The effects of Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+} ions on the HA of lectin were also evaluated in the presence of ethylenediaminetetraacetic acid (EDTA) as a metal ion chelator to ensure the total absence of these ions in solution. EDTA was added at different concentrations ranging from 0.1−20 mM to CSRL solution. Lectin was serially diluted, and the HA was determined. Solutions of $CaCl_2$, $MnCl_2$, $FeCl_2$, $ZnCl_2$, and $MgCl_2$ were added to the final concentration of 40 mM, and the hemagglutination titer was evaluated after incubating for a standard time at room temperature,[24] using native rabbit erythrocytes.

Mitogenic Activity Assay. The mitogenic activity was determined as described by Ngai and Ng[6] and Wang et al.[25] White Balb/c or black C57 mice (20−25 g) were killed by cervical dislocation, and the spleens were aseptically removed. Spleen cells were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve and resuspended to 5×10^6 cells/mL in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units penicillin/mL, and 100 mg streptomycin/mL. The cells (7×10^5 cells per 100 μL per well) were seeded into a 96-well culture plate, and serial dilutions of the CSRL solution (concentrations ranging from 0.16 to 5 mg/mL; 100 μL per well) and Concanavalin A (a potent lectin from the jack-bean, with known mitogenic inducing capabilities; 1.25 μg/mL) in 100 μL of medium were added. The cells were incubated at 37 °C in a modified atmosphere of 5% CO_2 for 72 h, and then, a 10 μL of methyl [3H]-thymidine (0.25 kBq, GE Healthcare) was added. The cells were incubated for a further 16 h under the same conditions. The cells were then harvested with an automated cell harvester onto a glass filter, and the radioactivity was measured with a Beckman model LS 6000SC scintillation counter.

Antiproliferative Activity Assay. The antiproliferative activity of CSRL was determined as described by Wong and Ng.[26] Breast cancer

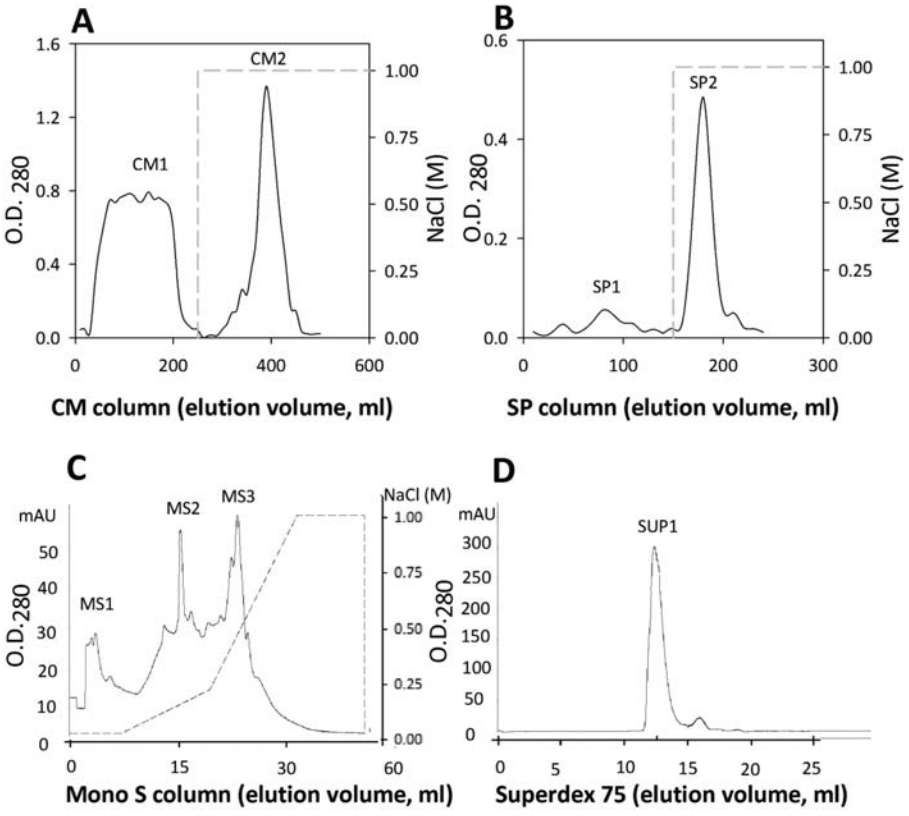

Figure 1. Purification of CSRL by chromatographic techniques. The elution patterns were for (A) the crude extract of chinook salmon roe on a CM-Sepharose column [fraction bound on the column (CM2) contained HA], (B) CM2 applied to the SP-Sepharose column (fraction SP2 was identified to contain HA), (C) SP2 applied to a Mono S column (fraction MS2 represented the peak containing HA), and (D) fraction MS2 applied to a Superdex 75 10/30 column to yield purified CSRL (SUP1).

cells MCF-7 and liver tumor cells Hep G2 were suspended in RPMI medium and adjusted to a cell density of 2×10^4 cells/mL. A 100 μL aliquot of this cell suspension was seeded to a well of a 96-well plate followed by incubation for 24 h. Different concentrations of CSRL (3.9−250 μM) in 100 μL of complete RPMI medium were then added to the wells and incubated for 48 h. After incubation, a 20 μL of 5 mg/mL MTT formazan [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] in phosphate-buffered saline was spiked into each well, and the plates were incubated for 4 h. The plates were then shaken at 2500 rpm for 5 min. The supernatant was carefully removed, and 150 μL of dimethyl sulfoxide was added in each well to dissolve the MTT formazan. After 10 min, the absorbance at 590 nm was measured by a microplate reader.

Assay of NO Production. The assay was conducted as described by Wong and Ng.[27] Male Balb/c mice (25−35 g) were each given 1 mL of sterile 3% (w/v) aqueous thioglycolate by intraperitoneal injection to stimulate macrophage formation. The mice were killed by cervical dislocation 3 days later. The peritoneal cavity was exposed, and peritoneal macrophages were collected by lavaging with PBS followed by centrifugation at 1600 rpm for 3 min. After the supernatant was discarded, lysis buffer was added to the cell pellet to destroy erythrocytes. The cells were washed with PBS and resuspended in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were seeded in a 96-well culture plate (2×10^5 cells/well) and left for 1 h before careful removal of the supernatant. A 200 μL aliquot of complete RPMI 1640 medium containing different concentrations of the lectin (0.625−20 μM final concentration) was added to each well and incubated with the macrophages for 24 h. NO was determined by measuring the amount of nitrite in the cell culture supernatant. After brief centrifugation, a 100 μL aliquot of cell-free culture medium from each well was allowed to react with 50 μL of Griess reagent (1% sulfanilamide in 5% H_3PO_4/0.1% naphthalene-ethylenediamine dihydrochloride) for 10 min. The absorbance at 540 nm was measured by using a BIO-RAD microplate reader, and the NO concentrations were calculated by using a standard calibration curve prepared by using different concentrations (1.5−200 μM) of sodium nitrite dissolved in the same medium. Lipopolysaccharide (LPS from *E. coli*, final concentrations from 6.25 to 250 μg/mL) was used as a positive control. Polymyxin B sulfate was used as a specific LPS inhibitor (final concentration 10 units/mL). Dexamethasone (final concentration, 10 μM) was used as a specific inhibitor of inducible nitric oxide synthase (iNOS).

Antifungal Activity Assay. The assay for antifungal activity toward *Rhizoctonia solani*, *Valsa mali*, *Helminthosporium maydis*, *Mycosphaerella arachidicola*, *Setosphaeria turcica*, and *Bipolaris maydis* was carried out in 100 mm × 15 mm Petri plates containing 10 mL of potato dextrose agar as described by Wong and Ng.[28] Aliquots containing 0.5 or 1.5 mg/mL of CSRL were used.

Statistical Analysis. All of the experiments were carried out in triplicates. Analysis of variance was carried out using Minitab 15.1.1.0. The significance of difference between means was obtained by Tukey's multiple comparison test ($P < 0.05$). Values are reported as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Purification of CSRL. The purification of CSRL was achieved utilizing its property of binding selectively to cationic media. A homogeneous lectin fraction was obtained by ion exchange

Table 1. Purification of Lectin from Chinook Salmon Roe[a]

fraction	volume (mL)	total protein (mg)	HA[b]	total HA[c]	specific activity[d]	purification fold	protein recovery (%)
crude extract	50	124.6	4096	4096000	32873.19	1.00	100
CM-Sepharose	50	75.72	8192	8192000	108188.06	3.29	60.77
SP-Sepharose	50	64.5	8192	8192000	127007.75	3.86	51.77
Mono S	5	0.97	16384	1638400	1689072.16	51.38	0.78

[a] The purification stages are described in section 2.3. [b] HA was expressed as a titer, the reciprocal of the highest twofold dilution exhibiting positive hemagglutination. The activity was determined using native rabbit erythrocytes. [c] Total hemagglutination titer (HA × volume). [d] Hemagglutination units per mg of protein (total HA/total protein).

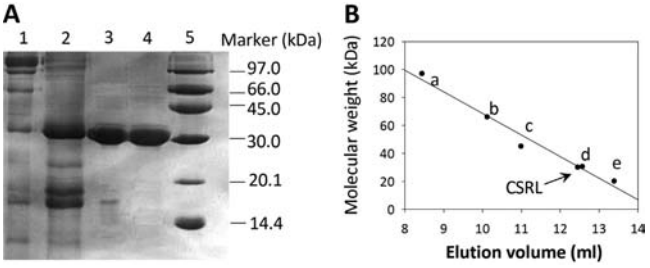

Figure 2. Determination of the MW of CSRL by SDS-PAGE and gel filtration on Superdex 75 10/300. (A) SDS-PAGE results. Lane 1, crude extract of salmon roe; lane 2, SP Sepharose bound fraction; lane 3, fraction MS-2 from Mono S column; lane 4, purified CSRL from the peak SUP-1; and lane 5, protein ladder. (B) Determination of the MW of CSRL by gel filtration on Superdex 75 10/300. CSRL had a corresponding MW of 30 kDa. The markers included (a) phosphorylase b (97 kDa), (b) BSA (66 kDa), (c) ovalbumin (45 kDa), (d) carbonic anhydrase (30 kDa), and (e) soybean trypsin inhibitor (20 kDa).

chromatography on CM-Sepharose and SP-Sepharose media, followed by ion exchange chromatography by FPLC on a Mono S column.

The chromatogram from the ion exchange chromatography on CM-Sepharose is shown in Figure 1A. The unbound fraction (CM1) did not contain any HA, while the bound fraction (CM2) contained HA. The CM-Sepharose bound fraction (CM2) eluted with 1 M NaCl was subsequently applied to a SP-Sepharose column, which resulted in two peaks with the second (SP2) peak as the major peak. The elution profile is shown in Figure 1B—the main HA was found in peak SP2. Fraction SP2 was then loaded on a Mono S column. Three main peaks were produced (Figure 1C). Fraction MS2 from the Mono S column with HA was finally loaded on a Superdex 75 10/300 GL column, and a main peak SUP1, which constituted purified CSRL, appeared (Figure 1D). The purification and specific activity of CSRL was increased 3.3-, 3.9-, and 51.4-fold and 2-, 2-, and 4-fold, respectively, after CM-Sepharose, SP-Sepharose, and Mono S (Table 1).

The purified lectin exhibited one band with an apparent MW of 30 kDa in SDS-PAGE (Figure 2A). Upon gel filtration using a Superdex 75 column, it was eluted as a sharp peak with the same MW (Figure 2B). The sequence of the first 30 N-terminal amino acids is shown in Figure 3A. Sequence alignment uncovered a high homology of CSRL with other fish lectins, including CSL1 (a trimer of identical 31.2 kDa subunits) from chum salmon (*Oncorhynchus keta*) eggs,[12] STL1 (a noncovalently linked trimer consisting of the 31.4 kDa subunits),[29] WCL (a trimer of identical 35 kDa subunits),[30] and a rhamnose-binding lectin (*Esox lucius*).[31] Thus, CSRL is subsumed into the L-rhamnose-binding lectin family.

Besides CSRL, there are other lectins reported from chinook salmon roe such as SEL 24K, SEL 26Ka, SEL 26Kb, and a 122 kDa one.[14,15,32] The first three lectins are closely related proteins, and their cDNAs were recently reported.[15,32] The 24 kDa lectin SEL 24K selectively binds galactose/rhamnose. It contains 16 cysteine residues with a specific disulfide bond pattern and has been crystallized.[15,32] Voss and colleagues have purified a lectin of about 122 kDa.[14] It had about 4-fold of the molecular weight estimated in the present study, and it is likely to be a tertiary macromolecule structure consisting of four lectin subunits.[14]

It is worth noting that there is a sequence difference between CSRL and other fish lectins with a MW around 20−25 kDa, including SEL 24K (Figure 3B). This supports the belief that multiple L-rhamnose-binding isolectins have diverged by gene duplication and exon shuffling, which may have various biological roles in each species.[30]

HA of CSRL. Rabbit and bovine erythrocytes were agglutinated by CSRL but not sheep and chicken erythrocytes (data not shown). The treatment of native rabbit erythrocytes with trypsin and Pronase increased the HA of CSRL by 16- and 64-fold, respectively. Bovine erythrocytes were agglutinated to a lesser degree by CSRL, with native erythrocytes having a HA value of 4, but the trypsin- and Pronase-treated erythrocytes had much higher HA of 8192 and 2048, respectively.

Lectins react with erythrocytes by virtue of the presence of carbohydrate configurations on the surface of the erythrocytes or antigenic determinants of the human blood group substances.[33] Voss et al.[14] reported that the HA of chinook salmon ova crude extract was specific for rabbit and human type B erythrocytes but not for sheep, human type A, and O erythrocytes. Similarly, Kamiya et al.[11] found that chum salmon ova had the ability to agglutinate rabbit erythrocytes but not sheep, horse, and human A, B, and O erythrocytes. As compared to the HA reported by Voss et al.[14] and Kamiya et al.[11] toward rabbit erythrocytes, the CSRL in the present study had a higher HA.

Shiina et al.[12] reported three isoforms of a lectin from chum salmon eggs and demonstrated that the specificity of the lectin toward erythrocytes can differ, depending on the lectin isoform. This may indicate that environmental factors and/or the purification system employed can potentially generate different lectin isoforms with different properties.

The lectins from chinook salmon ova and chum salmon ova have similarities in terms of the HA that are specific for rabbit erythrocytes. In fact, rabbit erythrocytes appear to require only a small amount of salmon roe lectin to become agglutinated. It is difficult to compare the HA of coho salmon egg lectin as the authors chose to use a modified adsorption test in which SDS-PAGE instead of the conventional hemagglutination test was used to detect the protein.[13]

Figure 3. Alignment of amino acid sequences of CSRL and other fish lectins. Numerals on the left are position numbers of amino acids. GenBank numbers of the proteins are as follows: CSL1 (a trimeric lectin from chum salmon eggs), P86177.1; STL1 (a trimeric lectin from steelhead trout eggs), NP_001117667.1; WCL1 (a trimeric lectin from white-spotted charr eggs), BAB83628.1; ELL (*E. lucius* lectin), ACO14273.1; SEL_24K (a 24 kDa lectin from chinook), ABN09236; STL3 (a rhamnose binding lectin STL3 from *Oncorhynchus mykiss*), NP_001117669.1; CSL3 (a dimeric lectin from chum salmon eggs), P86179.1; SSL-1 (a rhamnose-binding lectin from *Salmo salar*), NP_001134213.1; Stl2 (a rhamnose binding lectin STL2 from *Oncorhynchus mykiss*), NP_001117668.1; CSL2 (a dimeric lectin from chum salmon eggs), P86178.1; and IFL (a rhamnose-binding lectin from *Ictalurus furcatus*), ADO28323.1.

Table 2. Results Obtained after the Hemagglutination Inhibition Assay Was Performed on Chinook Salmon Roe Crude Extract Using Various Carbohydrates[a]

well no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
							concentrations of sugar in mM/l							
sugar	200.00	100.00	50.00	25.00	12.50	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	0.02
							monosaccharides							
L-rhamnose	−	−	−	−	−	−	−	−	−	−	−	−	+	+
D-galactose	−	−	−	−	−	−	−	+	+	+	+	+	+	+
D-fucose	−	−	−	−	−	−	−	+	+	+	+	+	+	+
L-arabinose	−	−	−	−	−	−	−	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
							disaccharides							
maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
							polysaccharides							
melibiose	−	−	−	−	−	−	−	+	+	+	+	+	+	+
raffinose	−	−	−	−	−	−	−	+	+	+	+	+	+	+
N-acetyl glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] +, hemagglutinating activity; −, no hemagglutinating activity. Untreated chinook salmon roe crude extract had a hemagglutination titer of 4096 (12), tested using native rabbit erythrocytes.

The hemagglutination ability of CSRL is also similar to the activity reported by Hosono et al. [34] for catfish roe lectin, which agglutinated rabbit, human B, and bovine erythrocytes, but not human (A and O), horse, sheep, and chicken erythrocytes. Treatment of rabbit and bovine erythrocytes with trypsin and Pronase led to an increase in agglutination activity of CSRL. Treatment of the sheep and chicken erythrocytes in the present study with trypsin and Pronase did not change their agglutinating behavior, and no hemagglutination was observed. Hosono et al.[34] found that treating human A and human O erythrocytes with trypsin or Pronase led to strong agglutination by catfish roe lectin in addition to increasing the agglutinating activity of human B erythrocytes. This was not the case for CSRL and could be due to the sugars on the surface of the sheep and chicken red blood cells not having any affinity to agglutination by CSRL.

Inhibition of the HA of CSRL. L-Rhamnose was the strongest monosaccharide inhibitor and was 31 times more potent than D-galactose, D-fucose, and L-arabinose, which were also good

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Table 3. Hemagglutinating Activity of CSRL after Incubation at Different Temperatures for Different Time Periods (n = 3)

	hemagglutination titer (no. of wells)[a]				
temperature (°C)	5 min	10 min	20 min	30 min	60 min
30	4096 (12)	4096 (12)	4096 (12)	4096 (12)	4096 (12)
40	4096 (12)	4096 (12)	4096 (12)	4096 (12)	4096 (12)
50	4096 (12)	4096 (12)	4096 (12)	4096 (12)	4096 (12)
60	4096 (12)	4096 (12)	4096 (12)	4096 (12)	4096 (12)
70	4096 (12)	4096 (12)	4096 (12)	4096 (12)	4096 (12)
80	2048 (11)	1024 (10)	512 (9)	512 (9)	64 (6)
90	64 (6)	32 (5)	4 (2)	4 (2)	0

[a] Values in parentheses stand for numbers of wells of culture plates showing hemagglutination. Untreated CSRL had a hemagglutination titer of 4096 (12).

inhibitors (Table 2). The MIC of L-rhamnose was 0.10 mM, while for D-galactose, D-fucose, and L-arabinose, the MIC was 3.13 mM. Polysaccharides that were able to inhibit the HA of CSRL included melibiose and raffinose, which also had a MIC of 3.13 mM. Melibiose and raffinose have the same hydroxyl group orientation at C2 and C4 of the pyranose ring similar to the structure of L-rhamnose.

The HA of CSRL was not inhibited by other sugar configurations such as D-fructose, D-xylose, D-glucose, D-mannose, maltose, and lactose, even at a concentration of 200 mM. The inhibitory effect of sugars on CSRL is similar to the activity reported for other salmon gonads lectins. L-Rhamnose was the most potent monosaccharide inhibitor for chum salmon lectin.[12] Melibiose, raffinose, L-arabinose, D-galactose, and D-fucose, which possess the same hydroxyl group orientation at C2 and C4 of the pyranose ring structure of L-rhamnose, also showed inhibitory effects. Yousif et al.[13] reported that simple sugars (D-galactose, D-galactosamine, or L-rhamnose) resulted in total inhibition of the coho salmon egg lectin, preventing adsorption of the molecule to *Aeromonas salmonicida* cells. In contrast, the addition of D-mannose, D-glucose, *N*-acetyl D-galactosamine, and D-mannosamine had no inhibitory effect on the binding ability of this lectin. Most fish egg lectins that have been characterized so far fall into the rhamnose-binding lectin (RBL) family,[35] and it appears that CSRL in the present study shares the same characteristics.

Effect of Temperature on the HA of CSRL. CSRL was stable after exposure to temperatures ranging from 30 to 70 °C for up to 1 h (Table 3). The HA of CSRL was decreased by 50% after 5 min at 80 °C, and only 3% of the activity remained after 60 min at 80 °C. Greater inhibition was found at 90 °C, with 3% HA found after 5 min of exposure and complete inhibition after 60 min of exposure.

In comparison to other fish egg lectins reported in literature, CSRL in this study was found to be relatively stable. Shiina et al.[12] found that it took at least 90 min to inactivate two isolectins from chum salmon eggs at 80 °C, while the third isolectin was inactivated within 60 min at the same temperature. Kamiya et al.[11] reported 75% reduction in HA of chum salmon ova lectin using trypsin-treated rabbit erythrocytes after heating the lectin at 80 °C for 15 min. On the other hand, Yousif et al.[13] reported that coho salmon egg lectin was very stable, as its bacterial (*A. salmonicida*) binding activity was unaffected by heating at 100 °C for 15 min.

A wide range of heat stability has been reported for lectins from different fish roes. For example, Hosono et al.[34] using human B erythrocytes found that catfish roe lectin had 3% and 1.5% HA after heating at 60 °C for 30 min and at 100 °C for 5 min, respectively. Jung et al.[36] reported a gradual decrease in the HA of skipjack tuna egg lectin toward human A erythrocytes between 40 and 70 °C, and a complete inhibition at 75 °C was found after 15 min of incubation. A more sensitive lectin was reported from grass carp roe, which lost half of its activity after incubation at 40 °C for 10 min, 75% of the activity disappeared upon incubation at 70 °C for 30 min, and the activity was completely abolished after exposure to 80 °C for 5 min.[22]

Effect of pH, Acid, and Alkali on the HA of CSRL. CSRL was stable in the pH range of 4−11 (data not shown). The HA of CSRL was completely eliminated following treatment with 0.1 M HCl (pH 1) for 1 h and reduced to a hemagglutination value of 4 at pH 12. While the effect of pH on the HA of lectins originating from salmon roe/ova has not been widely reported, other studies such as those undertaken by Jung et al.[36] and Ngai and Ng[6] have studied the effects of pH on the HA of fish egg lectins such as in skipjack tuna (KPL) and cobia, respectively. The HA of KPL was stable in a pH range between 6.0 and 8.5, but the activity was completely inhibited below pH 3.5 and above pH 10. Cobia egg lectin was stable between pH 4 and 10, and only 25% of the activity remained at pH 12. The activity of cobia egg lectin was completely abolished following treatment with 0.1 M HCl (pH 1) for 1 h. In addition, Tateno et al.[37] found that steelhead trout isolectins maintained their hemagglutination activities between a pH of 4 and 7. These diverse stability characteristics of lectins from different sources can be very useful in sourcing suitable lectins for certain biological functions and under specific environment conditions (pH/temperature).

Effect of Metal Ions on the HA of CSRL. After treatment with different concentrations of EDTA, there was no significant loss of CSRL HA. This demonstrates the cationic-independent activity of this salmon lectin, which is similar to the results reported by Kamiya et al.[11] on the properties of a lectin from chum salmon ova.

All cations tested in the form of chloride salts ($CaCl_2$, $MnCl_2$, $FeCl_2$, $ZnCl_2$, and $MgCl_2$) at a concentration of 10 mM had no effect on the HA of CSRL (data not shown). This is similar to the results observed for steelhead trout egg lectin,[37] grass carp roe lectin,[10] rudd roe lectin,[38] and olive rainbow smelt roe lectin.[39] Other fish egg lectins have been reported to be augmented in activity by the addition of metal ions.[6,40] Among the studies on salmon gonad lectins mentioned previously, only Kamiya et al.[11] reported the effect of metal ions, and our results are in agreement with their findings.

Mitogenic Activity. There was no mitogenic activity for CSRL (up to 5 mg/mL) in splenocytes from Balb/c mice, while Con A, a well-known mitogen, which served as a positive control, induced mitogenesis with a response of 10581.5 ± 5522.3 cpm at a concentration of 1.25 μg/mL (data not shown). The experiment was repeated using murine splenocytes obtained from C57 black mice. Similarly, CSRL did not induce a mitogenic response in splenocytes from C57 mice at concentrations up to 5 mg/mL, while Con A induced mitogenic response with a response of 1548.0 ± 103.2 cpm at 1.25 μg/mL (data not shown).

Several fish egg lectins have been reported to have mitogenic activity. For example, grass carp lectin induced a mitogenic response in murine splenocytes at a concentration of 688 nM[22] and cobia egg lectin exhibited maximal mitogenic response from mouse splenocytes at a concentration of 14 μM.[6] Krajhanzl et al.[41] postulated that the lectins from powan, roach, and perch roes, which manifested mitogenic or cytotoxic activity, was a result of mediation by macrophages or cytotoxins.

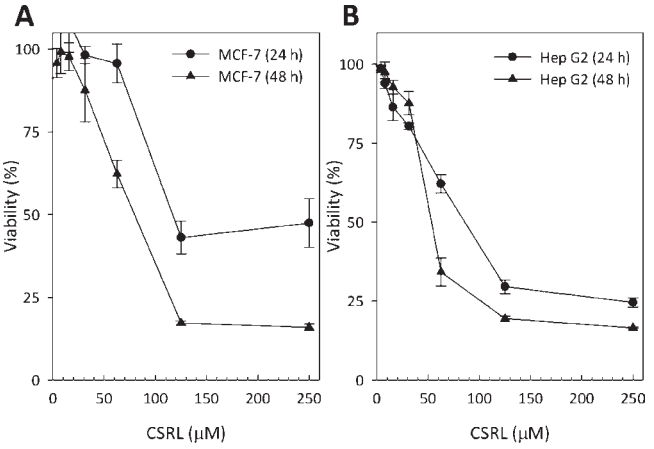

Figure 4. (A) Inhibition rate of CSRL toward the proliferation of MCF-7 tumor cells. (B) Inhibition rate of CSRL toward the proliferation of Hep G2 tumor cells.

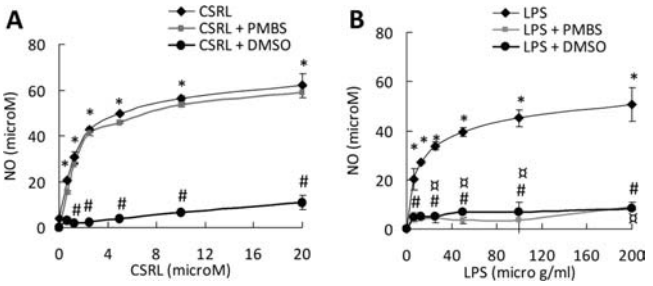

Figure 5. NO production by mouse peritoneal macrophages after different treatments. Macrophages (2×10^5 cells/well) were treated with (A) CSRL (final concentrations from 0.625 to 20 μM) or CSRL + polymyxin B sulfate (PMBS, final concentration 10 U/mL) or CSRL + dexamethasone (DMSO, final concentration 10 μM) and (B) LPS (final concentrations from 6.25 to 250 μg/mL) or LPS + PMBS (final concentration 10 U/mL) or EAPL + DMSO (final concentration 10 μM). The value of each data point represents the mean (SD of three independent experiments).

Another fish egg lectin that has been tested for mitogenic activity but did not exert this immunostimulatory behavior is the catfish roe lectin.[34] CSRL appears to share this characteristic in not having any mitogenic activity on murine splenocytes.

Antiproliferative Activity. CSRL reduced the proliferation of MCF-7 breast cancer cells (Figure 4A) and Hep G2 liver cancer cells (Figure 4B), in comparison to cells that were not treated with the lectin. At lectin concentrations of more than 125 μM, these cells showed lower levels of proliferation than the cells only control. For MCF-7 cells, a 57% decrease in the proliferation rate was obtained after 24 h at a lectin concentration of 125 μM, while after 48 h, an 82.8% decrease in the proliferation rate was achieved as compared to control cells. A higher CSRL concentration (250 μM) did not cause any further significant increase in the antiproliferative activity. For Hep G2 cells, a decrease in the proliferation rate of 70.5% was obtained after 24 h at lectin concentration of 125 μM, while after 48 h, the decrease in the proliferation rate increased to 80.6%. A higher CSRL concentration (250 μM) produced a slightly higher antiproliferation activity (75.5 and 83.5% after 24 and 48 h of exposure to CSRL, respectively) (Figure 4B). The IC_{50} values after 24 and 48 h of incubation were, respectively, 220 and 68 μM in the case of MCF-7 cells and 93 and 45 μM in the case of Hep G2 cells (data not shown). Normal liver cell line WRL68 was used as a control. A final CSRL concentration of 68 μM with 48 h of incubation was chosen to test its effect on WRL68 cells, with no detectable cell growth inhibition. This demonstrates that the tumor cells were more susceptible to CSRL than normal cells.

This is a novel finding in light of the fact that the antiproliferative activity of CSRL on these cells has not been reported previously and adds to the current knowledge that some lectins can selectively agglutinate tumor cells without any effect on their normal parental cells, which supports more research and interest in lectin-associated cancer investigations. A homodimer lectin, CSL3, was reported in chum salmon eggs, which manifested antitumor and apoptosis-inducing activities on Caco-2 (human epithelial colorectal adenocarcinoma) cells.[42] It has been suggested that the main mechanism of the antitumor activity of lectins lies in their ability to induce apoptosis via different pathways.[43] The results in this study reveal that CSRL is a potential antitumor agent, as it inhibited in a dose- and time-dependent manner the proliferation of two human tumor cells—human breast cancer MCF-7 cells and human liver cancer Hep G2 cells. The results from the present study represent the first report on the antiproliferative effect of a salmon roe lectin toward these two human cancer cells.

NO Inducing Activity of CSRL. CSRL stimulated iNOS expression and induced mouse peritoneal macrophages to produce at a CSRL concentration of 0.625 μM. A CSRL concentration of 20.5 μM induced an NO concentration of 62.0 μM (Figure 5A). Polymyxin B sulfate was used as a specific LPS inhibitor, while dexamethasone was used as a specific inhibitor of iNOS (Figure 5A). When polymyxin B sulfate was added to CSRL, NO production was similar to CSRL only, indicating that the concentration or contamination of LPS in the CSRL sample (if any) was negligible, while dexamethasone successfully suppressed the iNOS expression of CSRL (Figure 5B).

NO is synthesized from L-arginine mainly by nitric oxide synthase (NOS).[44] NO has been found to act as a potent cytotoxic agent in immune defense.[45] The high expression of NO in response to cytokines or to pathogen-derived molecules is considered to be an important component in the host defense.[46] Production by activated macrophages has been shown to inhibit tumor cell growth and tumor metastasis.[47] Up until now, few investigations have demonstrated the relationship between lectins and NO production. The present results represents one of the very few, if not the first report that demonstrates the ability of CSRL to induce NO production by macrophages, which may contribute partially to the antiproliferative activity toward the tumor cells described above.

Antifungal Activity. There was no antifungal activity found for CSRL against *R. solani*, *V. mali*, *H. maydis*, *M. arachidicola*, *S. turcica*, and *B. maydis* (data not shown). These results are similar to those found for cobia egg lectin.[6] The lack of antifungal activity of CSRL toward the six fungi tested in this study indicates that the lectin does not have an affinity toward them, neither by agglutination nor inhibition of growth. This is acceptable as some lectins demonstrate antipathogenic activities and some do not. For example, CSL1 and CSL3 have antipathogenic activity toward *A. salmonicida*, which is an aquatic Gram-negative bacterium causing the lethal disease of furunculosis in *Salmonidae*.[12] A mannose-specific, cobia egg lectin manifested antibacterial activity against *E. coli* but demonstrated no antifungal activity.[6]

To conclude, the present study revealed that a lectin purified and characterized from chinook salmon roe could act as a potential

antitumor agent, for it inhibited the proliferation of human breast cancer MCF-7 cells and human liver cancer Hep G2 cells and induced the production of NO by macrophages. This is the first report on such activity from chinook salmon roe. Lectins play important roles in many biological systems and are used increasingly in the therapy of human diseases. The knowledge gathered in this study about CSRL as a bioactive compound with activity toward human tumor cells points toward its potential use in biotechnological applications.

AUTHOR INFORMATION

Corresponding Author

*Tel: ++64 3 479 4994. Fax: ++64 3 479 7567. E-mail: aladin.bekhit@otago.ac.nz.

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ABBREVIATIONS USED

CSRL, chinook salmon roe lectin; EDTA, ethylenediaminetetraacetic acid; HA, hemagglutination activity; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide

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