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Macrophage-stimulating activities of newly isolated complex polysaccharides from *Parachlorella kessleri* strain KNK-A001

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ABSTRACT

Our previous studies demonstrated that the microalga *Parachlorella kessleri* (KNK-A001) has immunostimulatory activities, which were observed as an increase in natural killer (NK) cell activity in mice after intraperitoneal injection or as a protective effect on a virus-infected model shrimp after oral administration. In this study, we attempted to gain insight into the constituent substances of KNK-A001 that are responsible for the immunostimulatory activity. First, we obtained five polysaccharide fractions from KNK-A001 by DEAE anion exchange chromatography. Among the fractions, F5 showed the most potent induction of nitric oxide (NO) secretion in RAW264.7 cells, and both mRNA and protein expression levels of inducible NO synthase (iNOS) were increased in F5-treated RAW264.7 cells. A significant increase in the nuclear translocation of the p65 subunit of nuclear factor-kappa B (NF-κB) was observed in F5-treated RAW264.7 cells. F5 also induced the secretion of tumor necrosis factor (TNF)-α in RAW264.7 cells. Analysis using mitogen-activated protein (MAP) kinase inhibitors suggested that c-Jun N-terminal kinase (JNK) and p38 MAP kinase were mainly involved in F5-induced NO and TNF-α productions. The compositional analysis of F5 identified the main constituents as galactose, glucose, galacturonic acid, and mannose. Gel-filtration analysis suggested that molecular mass of F5 was approximately 400 kDa.

1. Introduction

The *Parachlorella kessleri* strain KNK-A001 is a green microalga taxonomically similar to *P. kessleri.* Although the microscopic morphological features of this microalga are indistinguishable from those of *Chlorella vulgaris*, it has a thick extracellular matrix that consists of polysaccharides, instead of the hard cell wall observed in *C. vulgaris*. In the course of our study to identify the functions of KNK-A001, we found that dry powdered KNK-A001 was a good value food for the Pacific oyster spat and the zooplankton rotifer *Brachionus plicatilis* [1]. Faecal analysis suggested that this was partly attributed to the easier digestion of KNK-A001 in those organisms compared with *C. vulgaris*, rather than to differences in nutrient value. Recent studies showed that the intraperitoneal injection of a KNK-A001 cell suspension into mice resulted in a significant increase in splenic natural killer (NK) cell activity against YAC-1 cells; the activity was much higher than that induced by *C. vulgaris*. Hence, it was suggested that KNK-A001 has potent *in vivo* immunostimulatory activity [2].

Kuruma shrimp (*Marsupenaeus japonicus*) is one of the most important aquatic organisms of aquacultural food resources in Japan. However, the farming of kuruma shrimp, as well as other commercially cultured shrimp species, has been severely impacted by white spot syndrome virus (WSSV) infection, which can cause almost complete mortality in the shrimp species within a few days of infection and often results in serious economic losses. As crustaceans do not have an acquired immune system, one of the most effective strategies for prophylaxis and the control of viral infections is the use of immunostimulants to activate the innate immune system. Consequently, the immunostimulatory effects of various substances, such as peptidoglycan, lipopolysaccharides, glucan, and other polysaccharides, have been extensively studied in fish and crustaceans [3–5] and the protective effects of some of these stimulants have been reported against WSSV infection [6].

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Based on this information, we recently investigated the protective effects of dry powdered KNK-A001 on WSSV-infected kuruma shrimp. Surprisingly, shrimp fed a diet containing 0.05% KNK-A001 showed a significantly higher survival rate than shrimps fed a control diet without KNK-A001. In addition, increased haemocyte cell density was observed in the haemolymph obtained from the shrimp fed 0.05% KNK-A001, and the superoxide anion producing activity of the haemocytes was also higher than that of the control [7]. These results suggested that KNK-A001 was a promising immunostimulant for use in shrimp farming to prevent or mitigate the impact of WSSV infection. However, the detailed mechanisms of the immunostimulatory activity of KNK-A001, especially the responsible substances, are completely unclear. As a significantly high level of mucus substances was detected in the suspension of dry-powered KNK-A001 in distilled water, we investigated the water-soluble fractions obtained from KNK-A001 and focused on the polysaccharides.

2. Materials and methods

2.1. Materials

KNK-A001 was cultured at 30°C in a glucose-based medium originally developed by Kaneka Co., Osaka, Japan. The dry powdered KNK-A001 was prepared with a drum dryer, as previously described [7]. DEAE-Toyopearl 650S was purchased from Tosoh Co., Tokyo, Japan. The mitogen-activated protein (MAP) kinase inhibitors (PD98059, SB202190, and 600125), which are specific inhibitors for extracellular-regulated kinase (ERK), p38 MAP kinase, and c-jun N-terminal kinase (JNK), respectively, were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). N^G-Monomethyl-L-arginine acetate (L-NMMA) was obtained from Dojindo Chemical Laboratories (Kumamoto, Japan). Anti-iNOS, anti-β-actin, anti-NF-κB p65, and anti-histone H3 antibody were purchased from Merck Millipore (Billerica, MA, USA), Abcam (Cambridge, UK), Cell Signaling Technology, Inc. (Danvers, MA, USA), and BioLegend (San Diego, CA, USA), respectively. Other chemicals were of the highest grade available commercially.

2.2. Extraction, isolation, and purification of polysaccharide fractions of KNK-A001

Dry powdered KNK-A001 was suspended in distilled water at 10 mg/mL and dialyzed with an 8000 MW cut-off against distilled water for 3 days at 4°C to remove low molecular weight substances. After dialysis, the suspension was autoclaved at 121°C for 15 min to extract the hot-water soluble fractions. To remove the insoluble materials, the suspension was centrifuged at 21,600*g* for 30 min at 4°C. The supernatant was applied to a column (2.5 × 40 cm) of DEAE-Toyopearl 650S previously equilibrated with distilled water. After elution with distilled water, the absorbed fractions were eluted with a linear gradient of NaCl (0–1.0 M). The sugar level in each fraction was monitored by a phenol-sulphuric acid method. To estimate the apparent molecular weight of the specific fraction (F5), the sample was applied to a column (1.6 × 93 cm) of Sepharose 4B previously equilibrated with distilled water, and eluted with distilled water. The elution was monitored by a phenol-sulphuric acid method. Dextrans of different molecular weights were used to construct the calibration curve.

2.3. Chemical composition analyses

The composition of neutral monosaccharides in each separated fraction obtained by anion-exchange chromatography was analysed with a high-performance liquid chromatography (HPLC) system (BioAssist eZ, Tosoh Co.) equipped with a PN-PAK C18 (3.0 × 75 mm) column using the ABEE method [8]. The content of phenolic compounds was estimated by the Folin-Denis method [9]. The protein level was measured by the RC DC™ protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Fourier-transformed infrared spectrum of F5 was measured by the KBr pellet method using the Nicolet Nexus 670NT FT-IR apparatus (Thermo Fisher Scientific Inc., MA, USA).

2.4. Cell culture

RAW264.7 (mouse macrophage) cells were obtained from American Type Culture Collection (Rockville, MD, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries, Ltd) supplemented with 10% foetal bovine serum (FBS), 100 IU/mL penicillin G, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Adherent RAW264.7 cells were harvested by mild scraping and collected by centrifugation (270*g* for 3 min at 4°C).

2.5. Cytotoxicity

The cytotoxic effect of the sample on RAW264.7 cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [8]. In brief, adherent RAW264.7 cells (5×10^4 cells/well in 96-well plates) were incubated with varying concentrations of the sample in the growth medium for 24 h and then MTT was added to the treated cells. After 30 min of incubation, the optical density of the MTT formazan reaction product was measured at 570 nm by using a Multiskan GO scanner (Thermo Fisher Scientific Inc., MA, USA).

2.6. Nitrite assay for the estimation of nitric oxide

Adherent RAW264.7 cells (5×10^4 cells/well in 96-well plates) were incubated with the indicated concentrations of various samples for 24 h at 37°C. After incubation, the nitrite levels in the culture medium of the treated cells were estimated by Griess assay. To examine the effects of three MAP kinase inhibitors, adherent RAW264.7 cells (5×10^4 cells/well in 96-well plates) were pre-incubated with each inhibitor at a final concentration of 10 μM for 1 h at 37°C in the growth medium. Subsequently, F5 was added to the treated cells at a final concentration of 500 μg/mL. After 24 h incubation, the nitrite levels in the culture medium were estimated.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Adherent RAW264.7 cells (5×10^4 cells/well in 96-well plates) were incubated with the indicated concentrations of various samples for 24 h at 37°C. After incubation, TNF-α levels in the culture medium of the treated cells were estimated by ELISA, as previously described [10]. The effects of MAP kinase inhibitors on the TNF-α production were examined by the same procedure as described above in the NO production.

2.8. RNA isolation, cDNA synthesis, and reverse transcription-polymerase chain reaction (RT-PCR)

Adherent RAW264.7 cells (2×10^6 cells/dish in a 35-mm dish) were treated with each sample at a concentration of 500 μg/mL at 37°C for 4 h. After incubation, the total RNA was extracted from the treated cells using TRIsure™ (NIPPON Genetics Co., Ltd, Tokyo, Japan) in accordance with the manufacturer's instructions. The extracted total RNA (2.5 μg) was reverse-transcribed into single-stranded cDNA by using the PrimeScript® 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan).