# Stimulatory effect of the sulfated polysaccharide ascophyllan on the respiratory burst in RAW264.7 macrophages

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## 1. Introduction

A brown alga (*Ascophyllum nodosum*) used as a raw material for the preparation of alginate, contains ascophyllan (xylofucoglycuronan) as a sulfated polysaccharide structurally similar but distinguishable from fucoidan [1,2]. Our previous studies have demonstrated that ascophyllan showed a growth-promoting activity on MDCK cells, while fucoidan was rather toxic to this cell line [3]. Furthermore, we found that ascophyllan had capability to induce the secretion of cytokine such as tumor necrosis factor-α (TNF-α) and granulocyte colony-stimulating factor (G-CSF) from mouse macrophage RAW264.7 cells [4]. Recent study has also demonstrated that ascophyllan induced much higher level of nitric oxide (NO) production from RAW264.7 cells than those induced by fucoidans isolated from *Fucus vesiculosus* and *Ascophyllum nodosum* [5]. Reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis revealed that the expression level of inducible NO synthase (iNOS) in ascophyllan-treated RAW264.7 cells was much higher than the level induced by fucoidans [5]. Electrophoretic mobility shift assay (EMSA) using infrared dye labeled nuclear factor-kappa B (NF-κB) and AP-1 consensus sequences suggested that ascophyllan strongly activated these transcription factors [5]. These *in vitro* studies suggest that ascophyllan has a potent activity to stimulate macrophages to induce the secretion of NO and cytokines through the activation of intracellular signaling pathways. In addition, antitumor activity of ascophyllan was observed in Sarcoma 180 ascites tumor-bearing mice model [6]. Since ascophyllan showed no significant direct cytotoxic effect on Sarcoma 180 cells, the antitumor activity was considered to be mainly exerted through the activation of host immune system [6].

Macrophages are multifunctional cells and play important roles in host defense system. After activation, they produce various inflammatory mediators and cytokines such as NO and TNF-α as mentioned above. Activation of macrophages is also associated with rise in oxygen consumption, leading to the production of reactive oxygen species (ROS), which play a pivotal role in the host defense against invading pathogens. This oxygen-related metabolic event is called the respiratory burst and NADPH oxidase is involved in the process as a main

enzyme. NADPH oxidase catalyzes one electron reduction of $O_2$ by NADPH to generate superoxide ($O_2^-$), which is secondary converted to other ROS. NADPH oxidase is a multicomponent enzyme consisting of at least two membrane-associated proteins ($gp91^{phox}$ and $p22^{phox}$), three cytosolic proteins ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$), and a small GTP-binding protein Rac [7,8]. When macrophages are exposed to certain stimulation, NADPH oxidase is activated to generate $O_2^-$ by association of three cytosolic components on the plasma membrane [9].

In the present study, we investigated the effect of ascophyllan on RAW264.7 cells in terms of ROS generation as well as the status of NADPH oxidase. Our results demonstrated that ascophyllan stimulated RAW264.7 cells to induce ROS generation through the activation of NADPH oxidase. This is the first report suggesting that a sulfated polysaccharide can stimulate macrophages to produce ROS.

## 2. Materials and methods

### *2.1. Materials*

Lipopolysaccharide (*Escherichia coli* 0111:B4; LPS), phorbol myristate acetate (PMA), polymyxin B and fucoidan were purchased from Sigma (St. Louis, MO, USA). PD98059, SB202190 and SP600125, which are specific inhibitors for extracellular-regulated kinase (ERK), p38 and c-jun $NH_2$-terminal kinase (JNK) mitogen-activated protein (MAP) kinase, respectively, standard dextrans (200, 40, 20, 12 and 5 kDa) were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Anti-$p47^{phox}$ (mouse) and anti-$p67^{phox}$ (mouse) polyclonal antibodies were purchased from Upstate, Serologicals Company (Lake Placid, NY, USA). Anti-β-actin polyclonal antibody was obtained from Abcam Inc. (Cambridge, USA). Antibodies for nonphosphorylated- and phosphorylated-p38, -JNK, and -ERK MAP kinases were purchased from Cell Signaling Thechnology, Inc. (Beverly, MA, USA). Other chemicals were of the highest grade commercially available.

*2.2. Preparation of ascophyllan from A. nodosum*

Ascophyllan was prepared from the powdered *A. nodosum* as separated fraction from fucoidan furaction as described previously [3, 4]. Ascophyllan solution was passed through an endotoxin-removal column (Detoxi-gel: Thermo Fisher Scientific Inc., Rockford, IL USA), and subsequently filtered through an endotoxin-removal filter (Zetapor Dispo: Wako Pure Chemical industries, Ltd, Osaka, Japan).

*2.3. Estimation of molecular mass*

Apparent molecular mass of ascophyllan was estimated by gel filtration chromatography [10, 11]. Briefly, the sample solution was applied to Superdex$^{TM}$ 200 10/300 GL column (1.0 cm × 30 cm; GE Healtheare, Piscataway, NJ, USA), eluted with 0.5 M sodium acetate buffer (pH 5.0) at a flow rate of 0.5 ml/min. Fractions of 0.5 ml/well were collected and detected with the phenol-sulfuric acid assay [12]. For the calibration, standard dextrans (200, 40, 20, 12 and 5 kDa) were applied to the same column at the same elute condition.

*2.4. Cell culture*

RAW264.7 (mouse macrophage) cells obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured at 37°C in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified atmosphere with 5% $CO_2$ and 95% air.

*2.5. Preparation of murine peritoneal exudate cells (PEC)*

6-week-old male ddY mice (Texam, Nagasaki, Japan) were intraperitoneally injected with 1

ml of 5% casein. After 3 days, the peritoneal exudate cells (PEC) were harvested, and adherent macrophages were prepared as described previously [13].

*2.6. Measurement of ROS*

In the chemiluminescence (CL) assays for the detection of ROS, we employed L-012 (Wako Chemical) as a highly sensitive chemiluminescence probe. The assay mixtures in CL assays consisted of, in order of addition, 80 μl of RAW264.7 cell suspension (final $10^6$ cells/ml), 10 μl of each sample (at final concentrations ranging from 10 to 1,000 μg/ml) or control Hanks' balanced salt solution (HBSS), and finally 10 μl of L-012 (final 100 μM). During the incubation, chemiluminescence emission was recorded by multilabel recorder Mithras LB940 (Berthold Technologies GmbH and Co. KG., Bad Wildbad, Germany).

*2.7. Superoxide ($O_2^-$) scavenging activity*

Superoxide scavenging activity of ascophyllan was measured as described previously [14], in which hypoxanthine/xanthine oxidase system was used as a source of $O_2^-$.

*2.8. Western blot analysis*

Plasma membrane fractions were prepared as described previously [14]. In brief, RAW264.7 cells ($10^7$ cells/ml) were incubated with ascophyllan (final 10 ~ 1,000 μg/ml) or PMA (final 0.1 μg/ml) in HBSS for 10 min at 37°C. The cells were collected by centrifugation (1,000×*g* for 5 min), and then suspended in 500 μl of extraction buffer consisting of 10 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM phenylmethyl sulfonyl fluoride, 0.1 mM EDTA, 10 mM leupeptin, 10 mM pepstatin and 2% Triton X-100. The cells were disrupted by sonication on ice. After centrifugation at 21,000×*g* for 15 min, the membrane pellet was washed with the extraction buffer to remove residual soluble elements by centrifugation at the same

condition. Protein concentrations in membrane fractions were determined with the BCA assay kit (BIO-RAD, Hercules, CA) using BSA as the standard. The extract was mixed with the equal volume of 2 x SDS-sample buffer (4% SDS, 100 mM Tris-HCl, pH 6.8, 20% glycerol) and incubated at 95℃ for 5 min. Aliquots of protein samples (20 μg) were separated on 12.5% of SDS-polyacrylamide gel and transferred to PVDF membranes (BIO-RAD, Hercules, CA). The membranes were blocked with 5% (w/v) skim milk in phosphate-buffered saline containing 0.1% (w/v) Tween 20 for overnight at 4 °C. Immunostaining of the blot was performed with anti-$p47^{phox}$, anti-$p67^{phox}$ (1:1,000) and Goat anti-rabbit IgG-horseradish peroxidase conjugate (Upstate Biotechnology, Lake Placid, NY, USA) (1:4,000). The blot was developed by ECL Plus western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA). For the analysis of MAP kinases, the whole-cell lysates were prepared from the treated RAW264.7 cells [5]. In brief, RAW264.7 cells ($10^7$ cells/well) were treated with 100 μg/ml of ascophyllan or PMA (final 0.1 μg/ml) in HBSS at 37℃. After 10 min incubation, the cells were washed two times with ice cold HBSS, and were lysed with 100 μl extraction buffer (10 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1% CHAPS, and 1% Triton X-100) containing 1% of protease inhibitor cocktail (Nakalai tesque Co., Kyoto, Japan). Samples containing 20 μg of proteins were subjected to 10 % of SDS-polyacrylamide gel, and western blot analysis using appropriate antibodies (1:1,000) against nonphosphorylated- and phosphorylated-p38, -JNK, and -ERK MAP kinases were carried out by the similar way as described above. To standardize the loaded protein levels, blotting with anti-β-actin antibody was also conducted at the same time.

*2.9. Statistical analysis*

All the experiments were repeated at least three times. Data were expressed as means ± standard deviation (S.D.). Tested groups were compared with appropriate controls using Dunnett's multiple comparison test. A difference was considered significant at $P < 0.05$.

## 3. Results

### *3.1. Estimation of molecular mass of ascophyllan by gel filtration chromatography*

To estimate molecular mass of ascophyllan, gel filtration chromatography was conducted. The apparent molecular weight of ascophyllan was estimated to be about 390 kDa based on the standard curve prepared from dextrans.

### *3.2. Stimulatory effects of ascophyllan on ROS production by RAW264.7 cells*

After addition of ascophyllan to RAW264.7 cells, L-012-enhanced CL responses were monitored. As shown in Fig. 1, significantly increased CL response was induced by ascophyllan. The effect of ascophyllan was concentration-dependent, and became the highest at 100 μg/ml. At 1,000 μg/ml, the CL response rather declined. Since the CL response induced by ascophyllan (100 μg/ml) was inhibited by superoxide dismutase (SOD), it was confirmed that the CL was due to superoxide. The CL response induced by 100 μg/ml of ascophyllan was comparable to that induced by 0.1 μg/ml of PMA, which had slightly different kinetics.

### *3.3. Superoxide-scavenging activity of ascophyllan*

Superoxide-scavenging activity of ascophyllan was examined by CL method. As a source of superoxide, we employed hypoxanthine (HPX)-xanthine oxidase (XOD) system. When XOD was added to the reaction mixture, a rapid CL response was observed (control), and the CL response was significantly reduced by ascophyllan in a concentration-dependent manner. In the presence of 1,000 μg/ml of ascophyllan, the integrated CL value during 2 min decreased to the similar level observed in the presence of 100 U/ml of SOD (Fig. 2).

### *3.4. Stimulatory effects of ascophyllan on murine PEC macrophages*

In the absence of any stimulants (control), ROS level in murine PEC macrophages was almost trace level (Fig. 3). Similar to RAW264.7 cells, increased ROS production was induced in murine primary macrophages by ascophyllan (100 μg/ml), and the increased ROS level was suppressed by SOD.

### *3.5. Effects of polymyxin B and diphenyleneiodonium (DPI) on ascophyllan-induced ROS production in RAW264.7 cells*

Polysaccharide samples isolated from seaweeds are often contaminated with trace amount of endotoxin. To check such possibility, effect of polymyxin B, an inhibitor of LPS, on ascophyllan-induced ROS generation in RAW264.7 cells was examined. As shown in Fig. 4A, polymyxin B showed no significant inhibitory effect on CL response in ascophyllan-treated RAW264.7 cells. In addition, LPS did not induce CL response in RAW264.7 cells (Fig. 4A). To ascertain whether ascophyllan-induced ROS was mediated by the activation of NADPH oxidase, RAW264.7 cells were pretreated with DPI, an inhibitor of NADPH oxidase. As shown in Fig. 4B, ascophyllan-induced CL was strongly inhibited by DPI.

### *3.6. Translocation of $p47^{phox}$ and $p67^{phox}$ to the plasma membrane in ascophyllan-treated RAW264.7 cells*

The macrophage NADPH oxidase is a multiprotein enzyme composed of the membrane-bound cytochrome b558 ($gp91^{phox}$ and $p22^{phox}$) and the cytosolic components ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and rac) [7,8]. The translocation of $p67^{phox}$ and $p47^{phox}$ subunits from the cytosol to the plasma membrane to associate with cytochrome b558 is an essential process for the formation of active NADPH oxidase [9]. In this study, we examined the membrane levels of $p67^{phox}$ and $p47^{phox}$ in ascophyllan-treated RAW264.7 cells. As shown in Fig. 5, the levels of $p67^{phox}$ and $p47^{phox}$ in the plasma membrane significantly increased in a concentration dependent manner after 10 min incubation with ascophyllan as compared to the control. The

treatment with PMA as a positive stimulant also induced the translocation of these subunits to the plasma membrane from cytosol.

### *3.7. Effects of MAP kinase inhibitors on the ascophyllan-induced ROS production in RAW264.7 cells*

MAP kinase signaling pathways are involved in various processes leading to macrophage activation. To investigate whether or not MAP kinases are involved in ascophyllan-induced ROS production in RAW264.7 cells, the effects of PD98059, SB202190 and SP600125, which are specific inhibitors for ERK, p38, and JNK MAP kinase, respectively, were examined. As shown in Table 1, SP600125 showed the most potent inhibitory effect on ascophyllan-induced ROS production in RAW264.7 cells. PD98059 showed the partial inhibitory effect but SB202190 had almost no effect. These results suggest that JNK MAP kinase mainly participated in the signaling pathways leading to ascophyllan-induced ROS production in RAW264.7 cells.

### *3.8. The activation of MAP kinases in ascophyllan-treated RAW264.7 cells*

To further investigate the involvement of MAP kinase signaling pathways in ascophyllan-induced ROS generation in RAW264.7 cells, the levels of phosphorylated ERK, p38, and JNK MAP kinases in ascophyllan-treated RAW264.7 cells were investigated. As shown in Fig. 6, dose-dependent increase in the level of phosphorylated JNK MAP kinases was observed in ascophyllan-treated RAW267.4 cells after 10 min incubation, whereas the expression of phosphorylated ERK did not show any significant differences between various doses of ascophyllan from 0 to 1000 μg/ml. And the level of p38 MAP kinases was very low as compared to those induced by PMA, which significantly increased the phosphorylation levels of all three MAP kinases.

*3.9. Comparison of the activities of ascophyllan, fucoidan, and dextran sulfate to induce ROS production*

To gain insight into structure-activity relationship, we also examined the activities of fucoidan and dextran sulfate to induce ROS production in RAW264.7 cells, and compared to that of ascophyllan. As shown in Fig. 7, 100 μg/ml of fucoidan induced ROS production, and the activity was slightly higher than that of ascophyllan, while 100 μg/ml of dextran sulfate showed no significant activity.

## 4. Discussion

Ascophyllan (xylofucoglycuronan) has similar but obviously distinct composition characteristics from those of fucoidans isolated from *A. nodosum* and *F. vesiculosus* [1,2]. Specifically, ascophyllan has fucose and xylose in about equimolecular proportion, whereas fucoidans have much higher ratio of fucose than of xylose. The sulfate levels of ascophyllan and fucoidans isolated from *A. nodosum* and *F. vesiculosus* were 9.6, 19.4, and 22.6%, respectively [4]. In addition, the apparent molecular mass of ascophyllan estimated by gel filtration chromatography was about 390 kDa, which is much higher than that of fucoidan (Sigma).

Our previous study demonstrated that ascophyllan induced NO and cytokines production from RAW264.7 cells through the activation of transcription factors nuclear factor-kappa B (NF-κB) and AP-1 [5]. In this study, we further investigated the effects of ascophyllan on macrophage activation. The results obtained in this study clearly indicated that ascophyllan is capable of inducing ROS generation in RAW264.7 cells through the activation of NADPH oxidase. CL analysis revealed that ROS level induced by ascophyllan was comparable to that induced by PMA, a potent stimulator of the respiratory burst activity. It has been reported that polysaccharides isolated from seaweeds are sometimes contaminated with endotoxins that have immunomodulatory effects [15]. To check this possibility, we used

polymyxin B (PMB) as an inhibitor for endotoxins and LPS [16]. No significant inhibitory effect of PMB on the ascophyllan-induced ROS generation was observed. More than that, exogenously added LPS did not induced ROS generation under the conditions used for ascophyllan analysis. Thus it is unlikely that endotoxin-like substances contaminated with ascophyllan may influence the activities of ascophyllan. Immunoblot analysis using specific antibodies revealed that ascophyllan promoted the translocation of cytosolic $p47^{phox}$ and $p67^{phox}$ subunits to the plasma membrane, which are essential steps for the activation of NADPH oxidase. This is the first report indicating that a sulfated polysaccharide can be a stimulator of the respiratory burst activity in macrophages.

Regarding seaweed-derived polysaccharides with stimulating activity on the respiratory burst of phagocytes, it has been reported that water-soluble extracts obtained from several seaweed species including three green algae, three brown algae, and one red alga showed potent stimulatory effect on the respiratory burst of fish (turbot) leucocytes, and the activity was mainly associated with polysaccharide fractions [17]. Among the samples tested, the polysaccharide fractions obtained from *Ulva rigida* were especially active [17]. Pre-incubation of the fish phagocytes with the extracts obtained from *U. rigida* and subsequent incubation with PMA resulted in higher respiratory burst activity than that in control cells stimulated with PMA alone. Thus, it seems that the extracts had a priming effects rather than a direct activation on the respiratory burst [17]. Further studies on the polysaccharide fractions obtained from *U. rigida* demonstrated that sulfated polysaccharides are the most effective stimulants on the respiratory burst of marine fish turbot leucocytes [18]. Interestingly, they also found that the stimulatory capacity was lost when the polysaccharides were desulfated, and resulfation of the molecules partially restored the activity. Based on these findings, they suggested that the stimulatory capacity of the polysaccharides is associated with the presence of sulfate groups. Similar to these findings, it has been shown that a sulfated polysaccharide obtained from the marine alga *Porphyra yezoensis* stimulated murine macrophages *in vitro* and *in vivo*, but this activity was lost after desulfation of the polysaccharide [19,20]. In addition to seaweed-derived polysaccharides, acid polysaccharides isolated from plant (*Tanacetum*

*vulgare*) have been reported to enhance ROS production in macrophages [21]. However, in those previous studies, ROS levels were analyzed after a few or even 24 h incubation with the polysaccharides samples, or analyzed on the combination with PMA. Regarding the processes leading to the respiratory burst activation, it is considered that there are priming and direct activation steps as distinct processes [22]. TNF-α, platelet-activating factor (PAF), and LPS showed priming effects, and preincubation with these agents prior to administering a stimulant resulted in much higher ROS generation than that induced by a stimulant alone [23-26]. High concentrations of these priming agents themselves can also lead to the production of $O_2^-$ [27]. Based on the results obtained in this study, it is considered that ascophyllan acts as a direct stimulant to activate macrophages, which may be distinct action mechanism from those of polysaccharides with priming effects described above.

It has been known that the major receptors for polysaccharides recognition in macrophages are Toll-like receptor 4 (TLR4), scavenger receptor class A (SRA), CD14, and complement receptor type 3 [28-32]. The binding of polysaccharides on these receptors induces several intracellular signal transductions, which can lead to macrophage activation. For instance, acetyl fucoidan induced NO production in RAW264.7 cells through TLR4, CD14, and SRA [33]. The analysis on MAPK signaling pathways demonstrated that acetyl fucoidan-induced NO production was mediated through activation of p38 MAPK and JNK, but not ERK. Similar to these findings, our previous studies suggested that ascophyllan-induced NO production in RAW264.7 cells was also mainly mediated through activation of JNK, but not ERK [5]. The study using specific MAPK inhibitors suggested that JNK and ERK MAPK, but not p38 MAPK, were involved in ascophyllan-induced ROS generation in RAW264.7 cells, although JNK MAPK inhibitor showed much stronger inhibitory effect than ERK MAPK inhibitor (Table 1). In addition, the western blot analysis showed that JNK MAPK was dose-dependently activated in ascophyllan-treated RAW264.7 cells, while the levels of activation of ERK and p38 MAPK were evidently lower than JNK MAPK. These results suggest that JNK MAPK plays a key role in ascophyllan-induced ROS generation, and ERK MAPK may also be partly involved in the activity.

In the case of serum-opsonized zymosan-induced superoxide production by bovine polymorphonuclear leukocytes (PMN), it has been reported that 100 μM of p38 MAP kinase inhibitor showed a potent inhibitory effect on the superoxide production, although it was used at higher concentration than our studies [34]. Probably the main MAP kinases responsible for the pathway leading to ROS generation in phagocytes may differ depending on the stimulants as well as cell types. Since our previous study demonstrated that JNK specific inhibitor showed the most potent inhibitory effects on ascophyllan-induced NO, it seems likely that JNK may commonly play a key role in the signaling pathways leading to both NO and ROS generation in ascophyllan-stimulated RAW264.7 cells. Further studies are required to clarify the intracellular signaling pathways leading to ROS generation as well as the specific receptors involved in ascophyllan-stimulated RAW264.7 cells.

Since fucoidan also induced ROS production in RAW264.7 cells, induction of ROS production in macrophages may be a common biological activity of sulfated fucans. However, dextran sulfate did not show the activity, suggesting merely sulfated polysaccharide is not sufficient to induce ROS production. Probably, entire structural characteristics of ascophyllan and fucoidan may be involved in the activity. Further studies are required to clarify this point.

In conclusion, our results suggested that ascophyllan is capable of inducing ROS generation in RAW264.7 cells through the activation of NADPH oxidase. Since ascophyllan immediately induced ROS generation after the addition, and the level of ROS induced by ascophyllan was comparable to the level induced by PMA, ascophyllan has a direct activation activity on the respiratory burst rather than the priming effect. JNK may play a key role in the signaling pathways leading to the respiratory burst activation induced by ascophyllan.

Table 1 Effects of MAP kinase inhibitors on ascophyllan-induced $O_2^-$ production from RAW264.7 cells.

| MAPK inhibitors | Level of $O_2^-$ (% of control) |
|---|---|
| SP 600125 | 21% ± 6.9% |
| PD 98059 | 84% ± 16.5% |
| SB 202190 | 103% ± 12.7% |

RAW264.7 cells (final $10^6$ cells/ml) were pre-incubated with ERK (PD98059), p38 (SB202190), or JNK (SP600125) MAP kinase inhibitors (final 10 μM) in HBSS for 10 min at 37°C, followed by the addition of 100 μg/ml of ascophyllan. After addition of L-012 (final 100 μM), the chemiluminescent responses during 60 min at 37°C were recorded immediately. Each value represents the means % of control ± standard deviation of triplicate measurements.

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**Figure legends**

Fig. 1. Time course of chemiluminescence (CL) responses of ascophyllan-treated RAW264.7 cells.

(A) RAW264.7 cells (final $10^6$ cells/ml) in HBSS were incubated with 0 (*), 10 (♦), 100 (▲) and 1,000 μg/ml (●) of ascophyllan or 0.1 μg/ml of PMA (○) at 37°C for 60 min. (B) RAW264.7 cells (final $10^6$ cells/ml) in HBSS were incubated with 100 μg/ml of ascophyllan in the presence (Δ) or absence (▲) of SOD (100 U/ml) at 37°C for 60 min. CL responses during 60 min were recorded immediately after addition of L-012 (final 100 μM).

Fig. 2. Superoxide scavenging activity of ascophyllan.

Effects of ascophyllan (final 0 ~ 1,000 μg/ml) or SOD (final 100 U/ml) on CL-responses caused by hypoxanthine (HPX)-xanthine oxidase (XOD) system were examined. The columns indicate the integrated CL-response intensity values during 2 min. Each value represents the means ± standard deviation of triplicate measurements.

Fig. 3. Time course of chemiluminescence (CL) responses of ascophyllan-treated mouse PEC primary macrophages.

Mouse PEC macrophages (final $10^6$ cells/ml) in HBSS were incubated with 100 μg/ml of ascophyllan in the presence (Δ) or absence (▲) of SOD (100 U/ml) or HBSS alone (control, *) at 37°C for 60 min. CL responses during 60 min were recorded immediately after addition of L-012 (final 100 μM).

Fig. 4. Effects of polymyxin B (PMB) and diphenyleneiodonium (DPI) on ascophyllan-induced CL responses in RAW264.7 cells.

(A) RAW264.7 cells (final $10^6$ cells/ml) were incubated with ascophyllan (final 100 μg/ml) (Δ, ▲) or LPS (final 0.1 μg/ml) (□, ■) in the presence (Δ, □) or absence (▲, ■) of polymyxin B (final 2 μg/ml) in HBSS at 37°C. (B) RAW264.7 cells (final $10^6$ cells/ml) were pre-incubated with (○) or without (●) DPI (final 10 μM) in HBSS at 37°C for 10 min, and then 100 μg/ml

ascophyllan was added. CL responses during 60 min were recorded immediately after addition of L-012 (final 100 μM). (*); control CL response.

Fig. 5. Levels of $p67^{phox}$ and $p47^{phox}$ in the plasma membrane fractions prepared from ascophyllan-treated RAW264.7 cells.

RAW264.7 cells (final $10^6$ cells/ml) were incubated with ascophyllan (final 10 ~ 1,000 μg/ml) or PMA (final 0.1 μg/ml) in HBSS for 10 min at 37°C. The plasma membrane fractions were prepared from the treated cells, and then subjected to SDS-PAGE and subsequent immunoblot analysis for $p67^{phox}$ and $p47^{phox}$ as described in the text.

Fig. 6. Effects of ascophyllan on phosphorylation of MAP kinases in RAW264.7 cells.

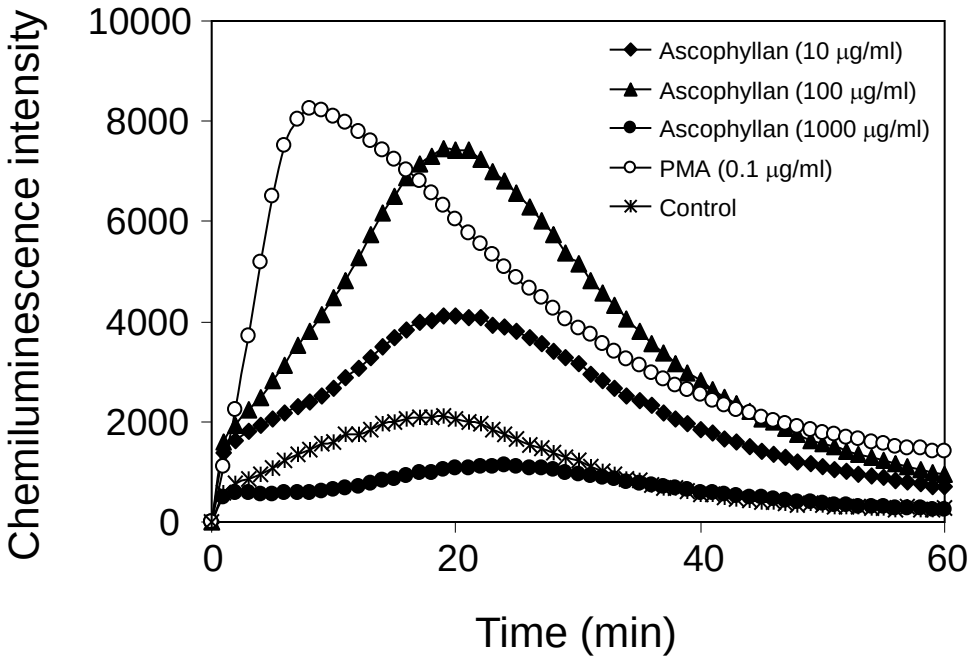
RAW264.7 cells were incubated with PMA (final 0.1 μg/ml) or varying concentrations of ascophyllan (final 10 ~ 1,000 μg/ml) for 10 min at 37°C. Cell lysates were prepared and subjected to SDS-PAGE and subsequent immunoblot analysis for JNK, phospho-JNK, ERK, phospho-ERK, p38, and phospho-p38 MAP kinases as described in the text.

Fig. 7. Comparison of the activities of ascophyllan, fucoidan, and dextran sulfate to induce ROS production in RAW264.7 cells.

RAW264.7 cells (final $10^6$ cells/ml) in HBSS were incubated with 100 μg/ml of ascophyllan, fucoidan, dextran sulfate, or HBSS alone (control) at 37°C for 60 min. CL responses during 60 min were recorded immediately after addition of L-012 (final 100 μM).

Fig. 1.

(A)


Chemiluminescence intensity
10000
8000
6000
4000
2000
0
0
20
40
60
Time (min)
Ascophyllan (10 μg/ml)
Ascophyllan (100 μg/ml)
Ascophyllan (1000 μg/ml)
PMA (0.1 μg/ml)
Control

(B)

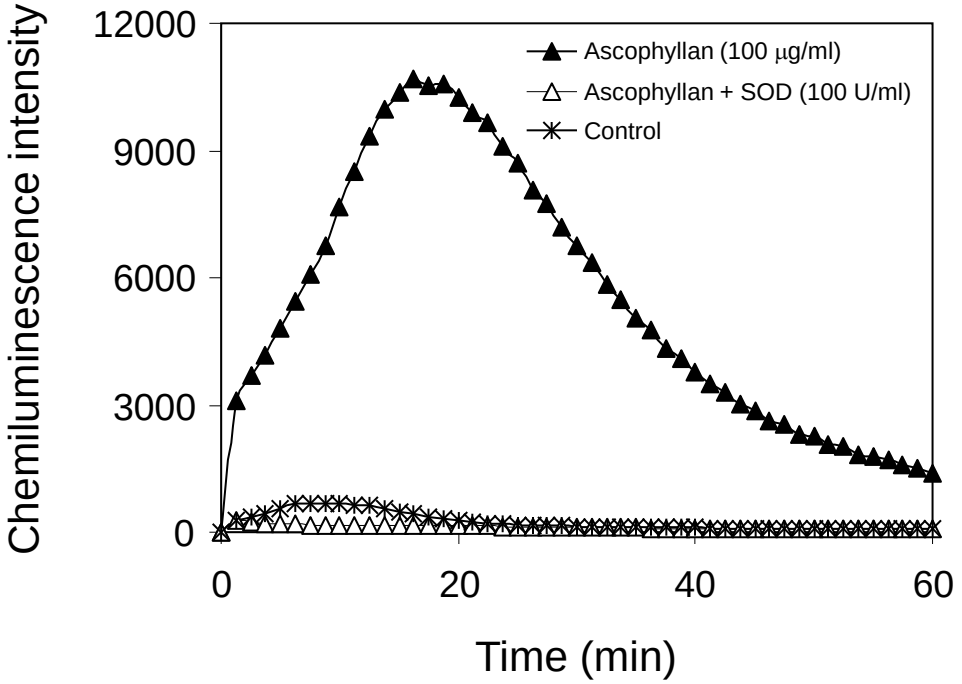

Chemiluminescence intensity
12000
9000
6000
3000
0
0
20
40
60
Time (min)
Ascophyllan (100 μg/ml)
Ascophyllan + SOD (100 U/ml)
Control

Fig. 2.

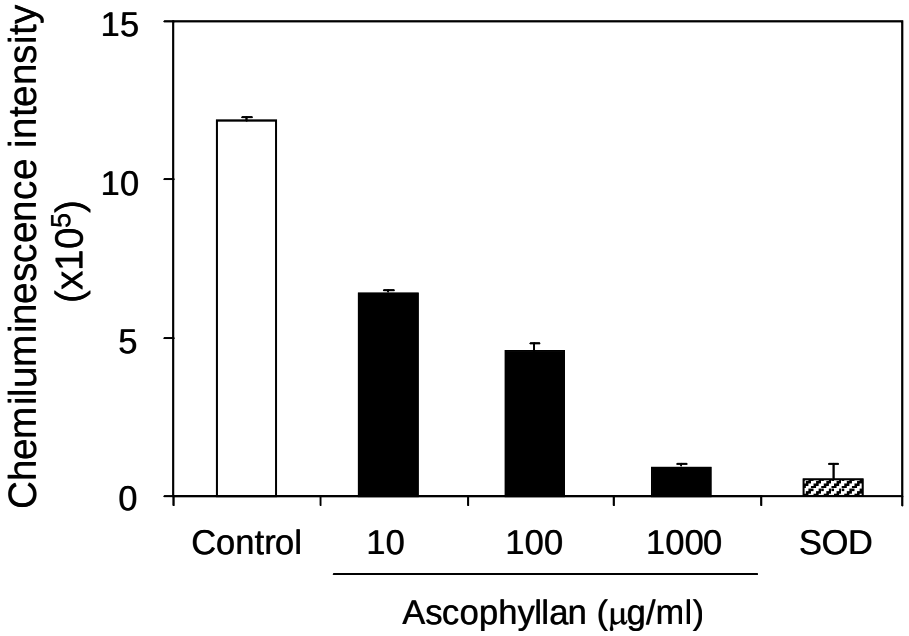

Chemiluminescence intensity
(x10^5)
15
10
5
0
Control
10
100
1000
SOD
Ascophyllan (μg/ml)

Fig. 3.

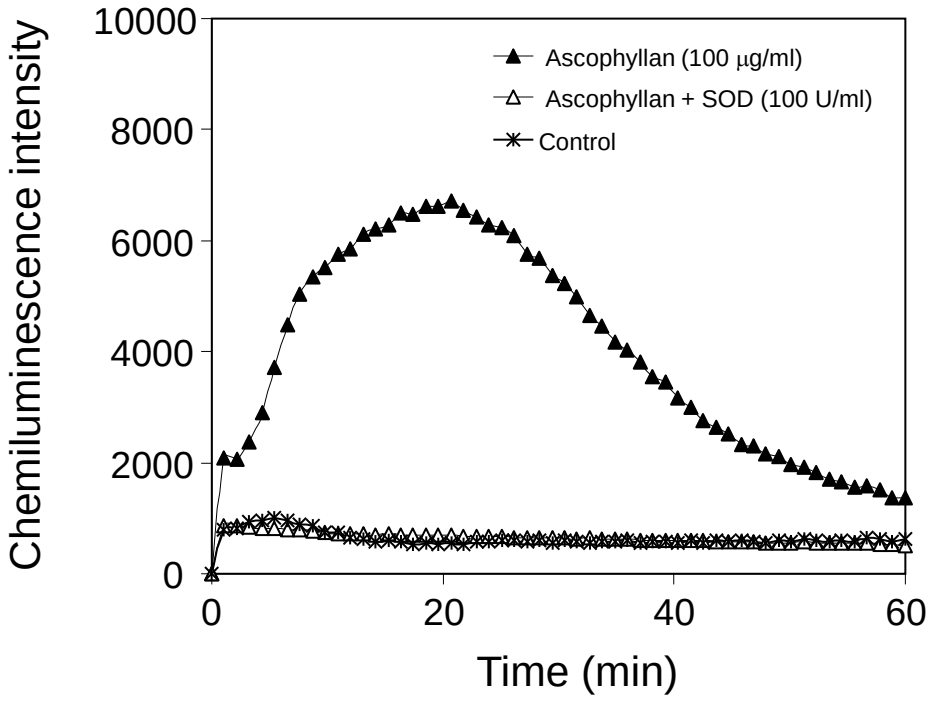
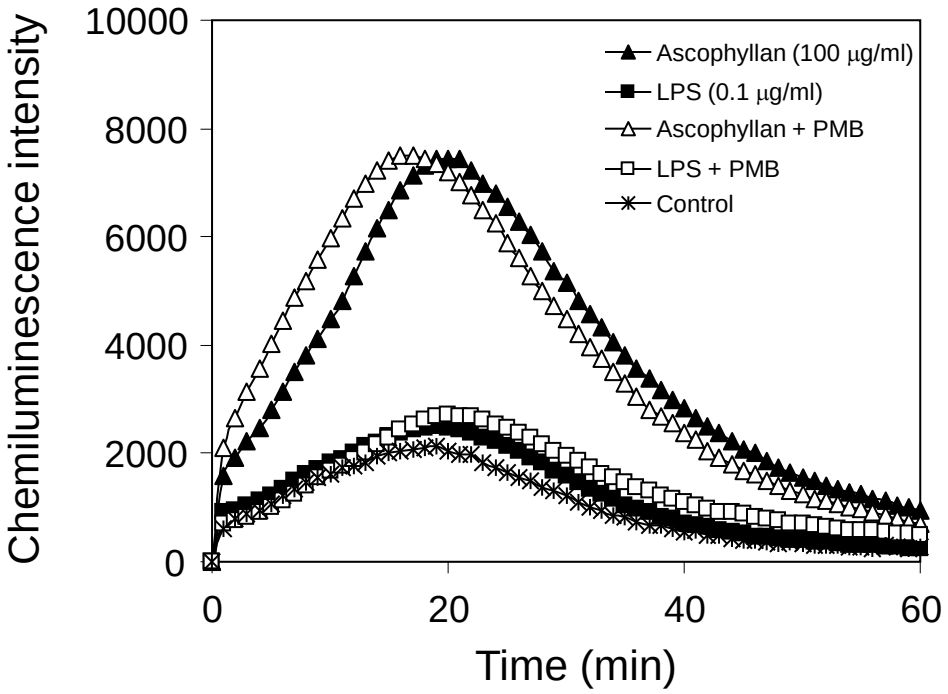

Chemiluminescence intensity
10000
8000
6000
4000
2000
0
0
20
40
60
Time (min)
Ascophyllan (100 μg/ml)
Ascophyllan + SOD (100 U/ml)
Control

Fig. 4.

(A)


Chemiluminescence intensity
10000
8000
6000
4000
2000
0
0
20
40
60
Time (min)
Ascophyllan (100 μg/ml)
LPS (0.1 μg/ml)
Ascophyllan + PMB
LPS + PMB
Control

(B)

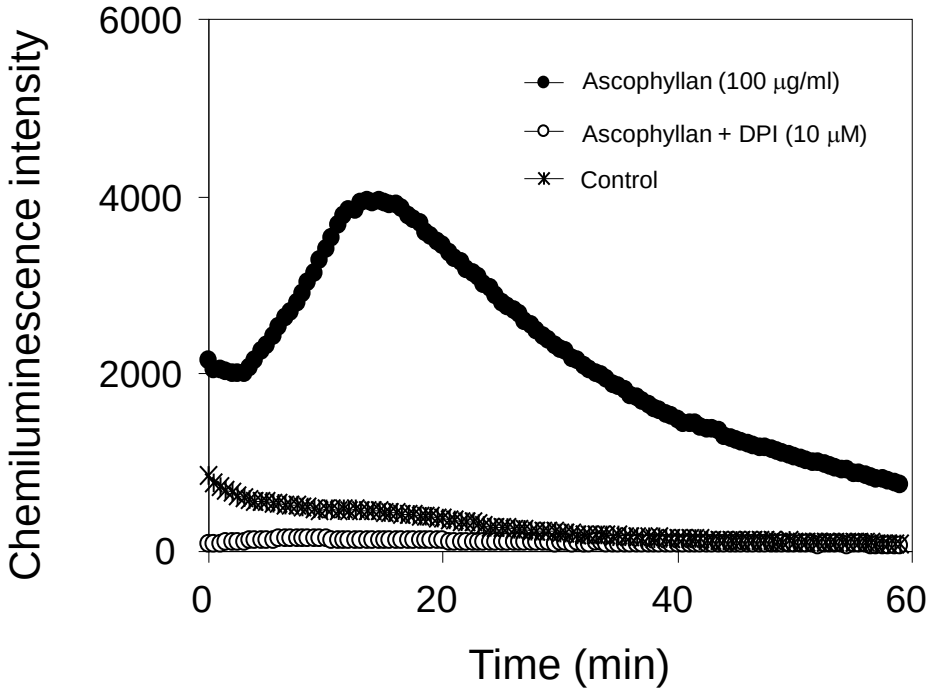

Chemiluminescence intensity
6000
4000
2000
0
0
20
40
60
Time (min)
Ascophyllan (100 μg/ml)
Ascophyllan + DPI (10 μM)
Control

Fig. 5.

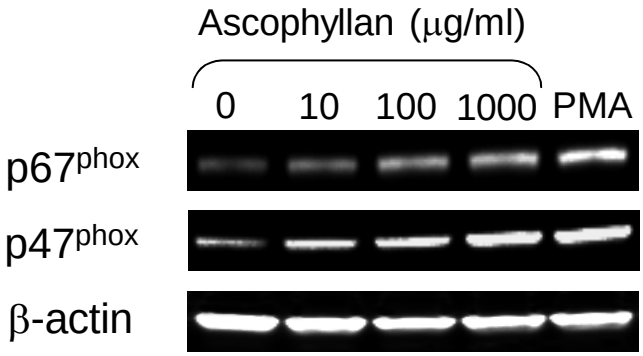


Fig. 6.

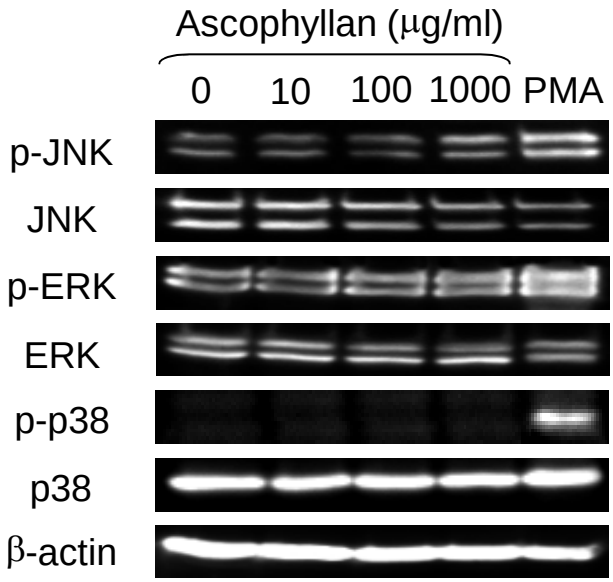
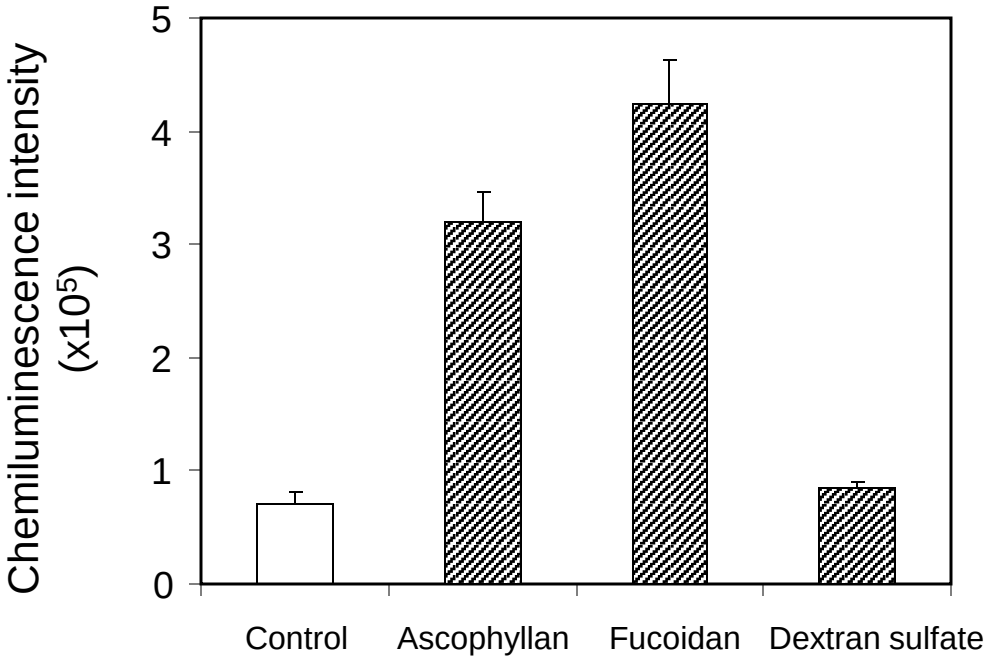

Ascophyllan (μg/ml)
0
10
100
1000
PMA
p-JNK
JNK
p-ERK
ERK
p-p38
p38
β-actin

Fig. 7.


Chemiluminescence intensity
(x10$^5$)
5
4
3
2
1
0
Control
Ascophyllan
Fucoidan
Dextran sulfate