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Structural characteristics and *in vitro* macrophage activation of acetyl fucoidan from *Cladosiphon okamuranus*

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Abstract We investigated a structural characteristics of acetyl fucoidan (CAF) isolated from commercially cultured *Cladosiphon okamuranus.* The CAF-induced macrophage activation and its signaling pathways in murine macrophage cell line, RAW 264.7 were also investigated. From the results of methylation analysis, CAF consisted of α-1→3 linked L-fucosyl residues and substituted sulfate and acetyl groups at C-4 on the main chain. CAF induced production of nitric oxide (NO), tumor necrosis factor-α and interleukin-6 in RAW 264.7 cells. Sulfate and acetyl groups of CAF involved in CAF-induced NO production. Neutralizing anti-Toll-like receptor 4 (TLR4), anti-CD14 and anti-scavenger receptor class A (SRA) but not anti-complement receptor type 3 monoclonal antibodies decreased CAF-induced NO production. The results of immunoblot analysis indicated that CAF activated mitogen-activated protein kinases (MAPKs) such as p38 MAPK, extracellular signal-regulated kinase (ERK)1/2 and stress-activated protein kinase/c-Jun *N*-terminal kinase (SAPK/JNK). SB203580 (p38 MAPK inhibitor) and SP600125 (SAPK/JNK inhibitor), but not U0126 (MAPK/ERK kinase 1/2 inhibitor) decreased CAF-induced NO production. The results suggested that CAF induced macrophage activation through membrane receptors TLR4, CD14 and SRA, and MAPK signaling pathways.

Keywords Acetyl fucoidan · *Cladoshiphon okamuranus* · Chemical structure · Macrophage · Nitric oxide · Cytokine

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Abbreviations

CAF	Acetyl fucoidan from *Cladosiphon okamuranus*
CR3	Complement receptor type 3
DMEM	Dulbecco's modified Eagle medium
iNOS	Inducible nitric oxide synthase
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase
NMR	Nuclear magnetic resonance
NO	Nitric Oxide
PMB	Polymyxin B
SAPK/JNK	Stress-activated protein kinase/c-Jun-*N*-terminal kinase
SRA	Scavenger receptor class A
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α

Introduction

Fucoidan is a sulfated polysaccharide found in the cell-wall matrix of brown algae. *Cladosiphon okamuranus* is an edible brown alga that is commercially cultured around the Okinawa Island, Japan. We have previously isolated a novel acetyl fucoidan (CAF) from *C. okamuranus* [1, 2], and which has been covered by a patent [3]. The yield of CAF was about 2.3% (*w*/*w*) from wet seaweed. The alginate was only one tenths of CAF content. Thus CAF is industrially producible. In fact, it has been prepared on an industrial scale from the alga and used as an additive to health foods, drinks and cosmetics in Japan. The structure of CAF is α-1→3 linked L-fucosyl residues and substituted with D-glucuronic acid at C-2 and sulfate groups at C-4 of the L-fucosyl residues, but the positions of acetyl groups were not determined [4, 5]. In aqueous solution, CAF molecules showed significant flow characteristics [6] which might be attributed to α-1→3 linked L-fucosyl residues.

There have been many reports that fucoidan from various brown algae have biological activities such as anti-tumor [7], anti-coagulant [8], anti-HIV [9] and inducing apoptosis [10]. CAF has been characterized to block the adhesion of *Helicobacter pylori* to human gastric cell line [11] and to ameliorate murine chronic colitis [12]. We have previously characterized that an oversulfated CAF induces apoptosis in U937 cells [13].

Macrophages are multifunctional cells and play a significant role in host defense. Macrophages phagocyte an invaded pathogen and produce inflammatory mediators and cytokines. Nitric oxide (NO), which is known to be inflammatory mediator, is produced by the activation of inducible nitric oxide synthase (iNOS) from L-arginine [14]. NO has been reported to play an essential role in the cytolytic function, killing of pathogens and tumoricidal action [15–17]. Cytokines have an important role in regulation of the inflammatory and immune responses [18]. Polysaccharides isolated from plants and algae have been reported to enhance macrophage activation [19–26]. Macrophage activation by polysaccharides is mediated through specific membrane receptors of macrophage. These receptors are pattern recognition molecules, which recognize foreign ligands such as carbohydrates, lipids and proteins during innate immune response. The major receptors reported for polysaccharides recognition in macrophages, are Toll-like receptor 4 (TLR4), CD14, complement receptor type 3 (CR3) and scavenger receptor (SR) [23–27]. TLR4 is transmembrane glycoprotein, which plays a critical role in innate and adaptive immune response [28, 29]. CD14 is a membrane-associated cell surface glycoprotein, which acts as co-receptor for TLR4 [30]. These two receptors are expressed on the surface of monocytes and neutrophils, and known as receptor for complex LPS, LPS binding protein and polysaccharides [30, 31]. CR3 is a heterodimeric transmembrane glycoprotein, which has been reported as leukocyte membrane receptor for β-glucans [32]. SR class A (SRA) I/II are trimeric transmembrane glycoproteins. SRA associates with cell adhesion, recognition and response to pathogens. SRA recognizes a wide variety of ligands, which include lipoprotein and polyanions [33]. Activation of these receptors is mediated by intracellular signaling pathways, resulting in macrophage activation [24–26]. Mitogen-activated protein kinases (MAPKs) are family of serine/threonine protein kinases that play a critical role in intracellular signaling. MAPK pathways regulate the cell survival, proliferation, differentiation, apoptosis and inflammation [34–36]. MAPK pathways are sequentially activated by phosphorylation, and then activated MAPKs lead to activation of transcription factors resulting in induction of various genes. MAPK family members, p38 MAPK, extracellular signal-regulated kinase (ERK)1/2 and stress-activated protein kinase/c-Jun-*N*-terminal kinase (SAPK/JNK) play an important role in macrophage activation by polysaccharides [24–26, 37–39].

In this study, we investigated the structural characteristics of CAF by methylation analysis. The CAF-induced macrophage activation and its signaling pathways in murine macrophage cell line, RAW 264.7 were also investigated.

Materials and methods

Materials and reagents

C. okamuranus, which was commercially cultured on nets at sea off the southern part of Okinawa Island on March in 2005, was used in this study. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Lipopolysaccharide (LPS) from *Escherichia coli* O2 was from Nacalai Tesque (Kyoto, Japan). Anti-iNOS rabbit IgG monoclonal antibody, anti-β-actin rabbit IgG polyclonal antibody and polymyxin B (PMB) were from Sigma (St. Luis, MO, USA). Neutralizing anti-TLR4 (rat IgG_{2a}), anti-CD14 (rat IgG_{2b}), anti-CR3 (rat IgG_{2b}) and anti-SRA type I/II (rat IgG_{2b}) monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), BD Pharmagen (San Diego, CA, USA), SouthernBiotec (Birmingham, AL, USA) and AbD Serotec (Raleigh, NC, USA), respectively. Rat normal IgG_{2a} and IgG_{2b} were from Biolegend (San Diego, CA, USA). Anti-phospho-p38 MAPK, anti-p38 MAPK,

anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-SAPK/JNK and anti-SAPK/JNK rabbit IgG monoclonal antibodies, and U0126 were from Cell Signaling Technology (Beverly, MA, USA). SB203580 and SP600125 were from BIOMOL International (Plymouth Meeting, PA, USA).

Preparation of CAF from *C. okamuranus*

Extraction and purification of CAF were carried out as described previously [2]. A wet seaweed sample (100 g) was homogenized with addition of double the weight of 0.05 M HCl and stirred at room temperature for 4 h to extract of CAF. The extract was centrifuged and the supernatant was filtered through Celite 545. The filtrate was neutralized with 0.1 M NaOH solution and precipitated by the addition of 2 volume of ethanol. The precipitate was dried *in vacuo*. The crude CAF was dissolved in 0.1 M $CaCl_2$ solution and passed through Celite 545. Filtrate was dialyzed against water for 24 h. The dialysate was deionized passing through a cation exchange column of Amberlite IR-120 (H^+) and then neutralized with 0.05 M NaOH solution, and then dialyzed against distilled water at room temperature for 24 h and then lyophilized.

Chemical methods

The total carbohydrate and uronic acid of CAF were analyzed by the phenol–sulfuric acid method and carbazole–sulfuric acid method, respectively. The sulfate content of CAF was measured turbidimetric method [40].

Determination of sugar compositions

The monosaccharides in the hydrolysate of CAF were determined using high-performance anion exchange chromatography (DX-500, Dionex Co., CA, USA), fitted with a column of Carbopack PA1 and pulsed amperometric detector. The column was eluted at flow rate of 1 mL/min at 35°C with 15 mM NaOH for neutral sugar and 100 mM NaOH/150 mM CH_3COONa for uronic acid.

Desulfation and deacetylatinon of CAF

Desulfation was performed by the solvotic desulfation method [41]. Briefly, CAF was converted into its pyridinium salt. The pyridinium salt was treated with mixture of dimethylsulfoxide/methanol (9:1, *v/v*) at 80°C for 4 h. After the mixture solution was dialyzed and then lyophilized. Deacetylation of CAF was performed by stirring in 50 mM NaOH/13.5 mM NaCl solution at room temperature for 13 h in an atmosphere of N_2. The solution was neutralized with 0.1 M HCl, dialyzed against, and then passed through Celite 545. The filtrate was lyophilized.

^{1}H-nuclear magnetic resonance (NMR) spectrum of CAF

^{1}H-NMR spectrum was recorded on a FT-NMR spectrometer (JNM-α500, JEOL Ltd., Tokyo, Japan) at 500.00 MHz, 60°C. The ^{1}H-NMR chemical shift was expressed parts per million (ppm) relative to internal sodium 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP, 0.00 ppm).

Methylation analysis

Native, and desulfated–deacetylated CAF were methylated by the Hakomori method, respectively [42]. The methylated CAF was hydrolysed with 2 M trifluoroacetic acid at 121°C for 1 h. The partially methylated sugars were reduced with $NaBH_4$, and then acetylated with acetic anhydride. The resulting partially methylated alditol acetates were analyzed by gas-chromatography (GC-17A, Shimadzu CO., Ltd., Kyoto, Japan) and characterized by gas chromatography-mass spectrometry (QP-5000, Shimadzu Co., Ltd., Kyoto, Japan).

Cell culture

RAW 264.7 cells (ATCC TIB-71) were obtained from American Type Culture Collection (Bethesda, MD, USA). Cells were grown in DMEM, which contained 10% (*v/v*) fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were plated and incubated at 37°C and 5% CO_2 atmosphere.

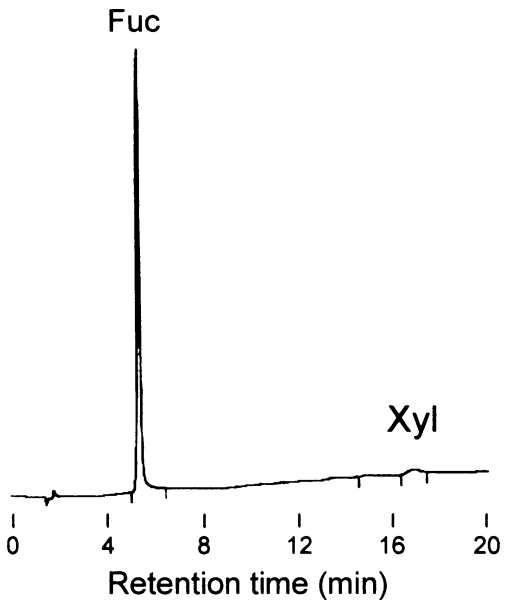

Fig. 1 Liquid chromatogram of hydrolysate of CAF

Table 1 Chemical compositions of CAF

	Total carbohydrate (%)	Uronic acid (%)	Compositions (molar ratio)				
			Fuc	Xyl	GlcA	Sulfate	Acetate
CAF	67.1	12.1	4.0	0.03	1.0	1.8	1.0

Measurement of NO production

NO production was determined by measuring the accumulation of nitrate in the culture supernatant using Griess reaction. RAW 264.7 cells were plated at 2.5×10^5 cells/well in 48 well culture plates, and then treated with CAF. The 100 μL of supernatants were obtained, and then mixed with 100 μl of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthy)-ethylenediamine dihydrochloride and 2% H_3PO_4) and incubated at room temperature for 20 min. After incubation, the absorbance of reaction mixture was measured at 550 nm with a microplate reader. The nitrate concentration in the supernatants was calculated with using a $NaNO_2$ standard curve.

Measurement of TNF-α and IL-6 production

RAW 264.7 cells were plated 2.5×10^5 cells/well in 48 well culture plates. The cells were treated with various concentration of CAF for 24 h, and then the culture supernatants were obtained. Concentrations of TNF-α and IL-6 in the supernatants were measured by commercial enzyme linked immunosorbent assay (ELISA) kits (Pierce biotechnology, Inc., IL, USA) according to the manufacture's instructions.

Inhibition of endotoxin

Possible contaminants of endotoxin (LPS or Lipid A-associated protein) in CAF were inhibited with PMB. CAF and LPS were treated with PMB (100 IU/mL) for 1 h and then used to activate RAW 264.7 cells. The NO production in RAW 264.7 cells was determined measuring the accumulation of nitrate in the culture supernatant using Griess reaction.

Effect of neutralizing anti-TLR4, anti-CD14, anti-CR3 and anti-SRA monoclonal antibodies on CAF-induced NO production in RAW 264.7 cells

Neutralizing anti-TLR4 (rat IgG_{2a}), anti-CD14 (rat IgG_{2b}), anti-CR3 (rat IgG_{2b}) and anti-SRA (rat IgG_{2b}) monoclonal antibodies were used for neutralizing TLR4, CD14, CR3 and SRA, respectively. Cells were pretreated with 10 μg/mL of neutralizing anti-TRL4, anti-CD14, anti-CR3 or anti-SRA monoclonal antibodies in the CO_2 incubator at 37°C for 2 h, and then treated with CAF (50 μg/mL) for 24 h. Rat normal IgG_{2a} and IgG_{2b} were used as isotype-matched control antibodies. The NO production in RAW 264.7 cells was determined measuring the accumulation of nitrate in the culture supernatant using Griess reaction.

Immunoblot analysis

RAW 264.7 cells were treated with CAF. The cells were scraped off the dish in phosphate buffered saline and spun at 2,000×*g* for 3 min. The cells were lysed with SDS sample buffer (125 mM Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and centrifuged at 15,000×*g* for 5 min. The protein content in cell lysates were measured by modified Lowry assay (DC protein assay, Bio-Rad Laboratories, Inc., CA, USA). An equal amount of proteins were separated by SDS-PAGE, then electro-transferred to PVDF membrane. The quantification of iNOS protein and analysis of p38 MAPK, ERK1/2, SAPK/JNK activation were monitored by immunoblot analysis using anti-iNOS, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-SAPK/JNK, and anti-SAPK/

Table 2 Methylation analysis of the native, and desulfated–deacetylated CAF

Derivatives	Deduced linkage pattern	Molar ratio	
		Native CAF	Desulfated–deacetylated CAF
2,3,4-tri-*O*-methyl-D-glucuronic acid	D-glucuronic acid-(1→	1.0	1.0
2,4-di-*O*-methyl-L-fucose	→3)-L-fucose-(1→	Trace	3.0
2-mono-*O*-methyl-L-fucose	→3,4)-L-fucose-(1→	3.0	0.2
4-mono-*O*-methyl-L-fucose	→2,3)-L-fucose-(1→	0.7	1.0

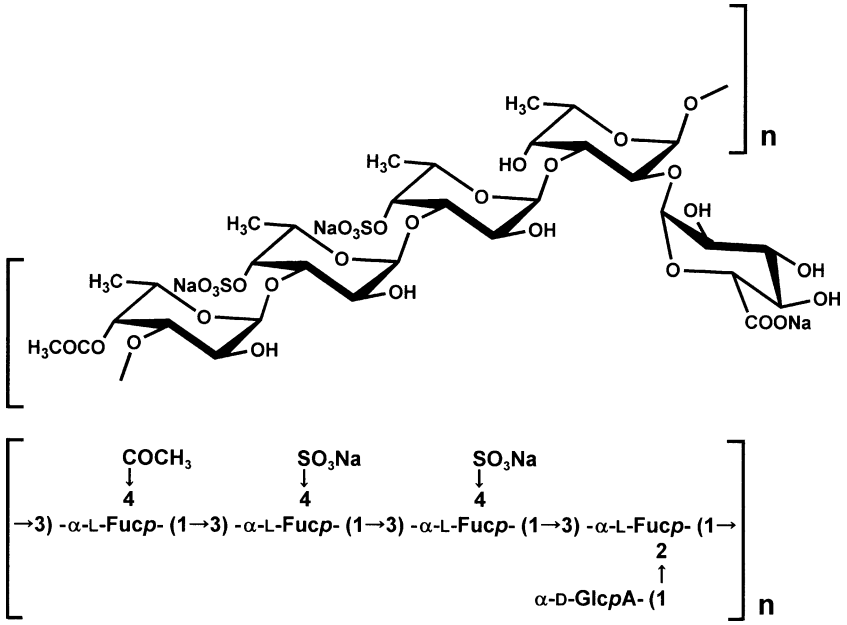

Fig. 2 The modified structure of CAF. CAF consisted of α-1→3 linked L-fucosyl residues, where D-glucuronic acid substituted at the C-2 and sulfate and acetyl groups at the C4

JNK antibodies. Immunoreactive bands were visualized with the chemiluminescence (ECL plus western blotting detection kit, GE Healthcare Biosciences).

Effect of MAPK inhibitors on CAF-induced NO production and MAPKs activation in RAW 264.7 cells

RAW 264.7 cells were pretreated with specific p38 MAPK inhibitor SB203580 (30 μM), specific MAPK/ERK kinase (MEK)1/2 inhibitor U0126 (10 μM) or specific SAPK/JNK inhibitor SP600125 (30 μM) for 1 h, and then treated with CAF (50 μg/mL). The measurement of NO production and MAPKs monitoring were determined by Griess reaction and immunoblot analysis, respectively.

Statistical analysis

Data are expressed as mean values±SEM and were analyzed using Student's *t* test or analysis of variance (ANOVA) followed by Dunnett's test for pair-wise comparison. The levels of significance were at a *P* value less than 0.05.

Results

Chemical compositions of CAF

Total carbohydrate and uronic acid of CAF were 67.1 and 12.1%, respectively. The sulfate content of CAF was estimated to be 13.5%. The liquid chromatogram of the

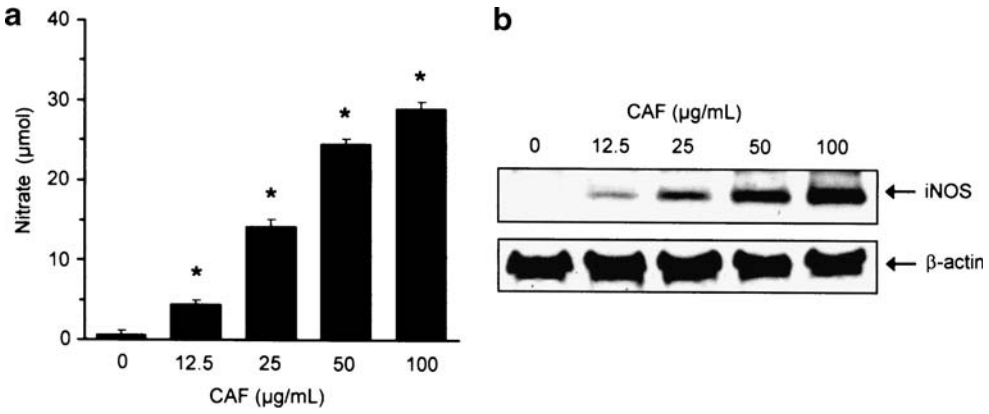

Fig. 3 Increase in NO production (**a**) and iNOS protein induction (**b**) in RAW 264.7 cells treated with CAF for 24 h. NO production was determined using Griess reaction. Data were means±SEM of three independent experiments. *Asterisks* show statistically significant difference from the control group ($p<0.05$). Cell lysates were prepared and subjected to SDS-PAGE and then immunoblot analysis for iNOS and β-actin. The equal loading in each lane was demonstrated by the similar levels of β-actin

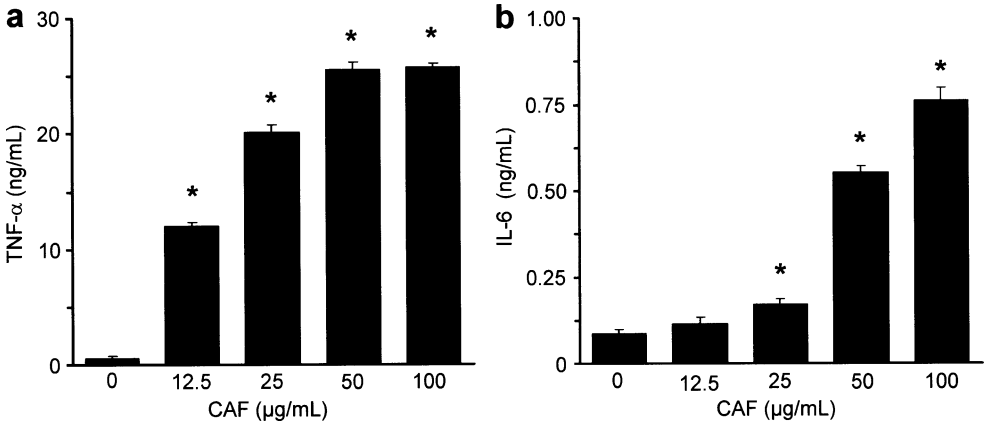

Fig. 4 Increase in TNF-α (**a**) and IL-6 (**b**) in RAW 264.7 cells treated with CAF for 24 h. TNF-α and IL-6 production were determined using ELISA. Data were means±SEM of three independent experiments. *Asterisks* show statistically significant difference from the control group ($p<0.05$)

hydrolysate of CAF indicated a major peak identical to L-fucose and small peak identical to D-xylose (Fig. 1), the molar ratio of which was 4.0:0.03. The D-glucuronic acid was also identified by liquid chromatography. The ratio of L-fucosyl residues and acetyl groups was estimated to be 4.0:1.0 by comparing the area of acetyl groups with that of methyl groups of L-fucose in the ^{1}H-NMR spectrum. CAF consisted of L-fucose, D-xylose, D-glucuronic acid, sulfate and acetate in the molar ratio of 4.0:0.03:1.0:1.8:1.0 (Table 1).

Methylation analysis

The primary structure of CAF was proposed by Sakai *et al.* [5], but the positions of the acetyl groups were not known. Native and desulfated–deacetylated CAF were methylated by the Hakomori method [42], and then reduced with $NaBH_4$ and acetylated with acetic anhydride. As a result of methylation analysis, 2,3,4-tri-*O*-methyl-D-glucuronic acid, 2-mono-*O*-methyl-L-fucose and 4-mono-*O*-methyl-L-fucose which originated from 1-linked D-glucuronic acid, 1,3,4-linked L-fucose, and 1,2,3-linked L-fucose, respectively were detected in native CAF (Table 2). Much more 2,4-di-*O*-methyl-L-fucose was detected in desulfated–deacetylated CAF than that of native CAF, but 2-mono-*O*-methyl-L-fucose was reduced. 2,3,4-Tri-*O*-methyl-D-glucuronic acid, 2,4-di-*O*-methyl-L-fucose, 2-mono-*O*-methyl-L-fucose and 4-mono-*O*-methyl-L-fucose in molar ratio of 1.0:3.0:0.2:1.0 were detected in desulfated–deacetylated CAF. The results indicated that sulfate and acetyl groups substituted at the C-4 of 1→3-linked L-fucosyl residues. Based on the results and references [4, 5], the modified chemical structure of CAF, as illustrated in Fig. 2, is proposed.

CAF-induced production of NO, TNF-α and IL-6 in RAW 264.7 cells

We investigated the effects of CAF on macrophage function, in RAW 264.7 cells. Firstly, we evaluate the effect of CAF on NO production of RAW 264.7 cells. The level of NO production can be used quantitative index of macrophage activation. As shown in Fig. 3a, the basal level of NO production from untreated cells was low. CAF significantly increased NO production in RAW 264.7 cells in a dose-dependent manner. To confirm that the increase in the NO production attributed to the induction of iNOS, we determined the iNOS protein levels by immunoblot analysis. CAF increased the iNOS protein level in RAW 264.7 cells in a dose-dependent manner (Fig. 3b). The β-actin protein level was unaffected by CAF. Furthermore, we evaluate the effect of CAF on inflammatory cytokine such as TNF-α and IL-6. CAF increased production of TNF-α and IL-6 in RAW 264.7 cells in a dose dependent manner (Fig. 4). The results demonstrated that CAF induced macrophage activation leading to production of NO, TNF-α and IL-6.

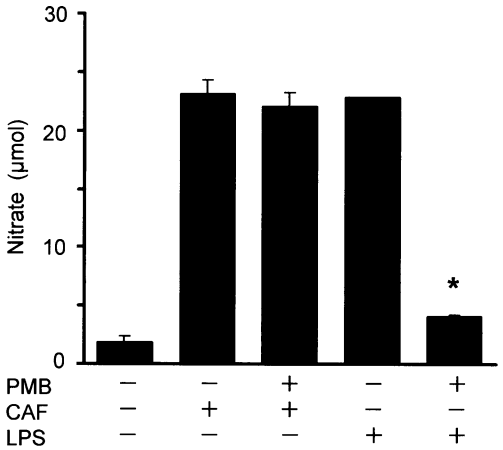

Fig. 5 Effect of PMB on CAF and LPS-induced NO production in RAW 264.7 cells. CAF and LPS were pretreated with PMB (100 IU/mL) for 1 h. RAW 264.7 cells were treated with PMB-treated CAF (50 μg/mL) or LPS (1 μg/mL) for 24 h. NO production was determined using Griess reaction. Data were means±SEM of three independent experiments. *Asterisk* shows statistically significant difference from the CAF or LPS treated groups ($p<0.05$)

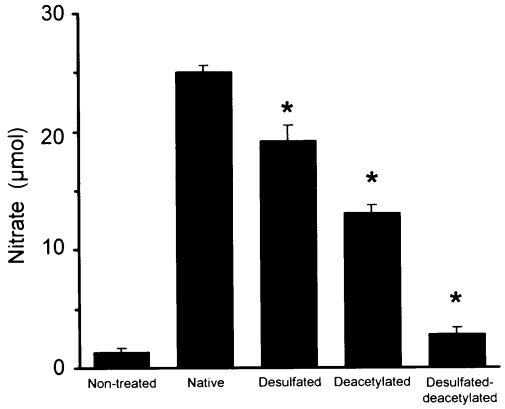

Fig. 6 Effect of desulfated, deacetylated and desulfated–deacetylated CAF on NO production in RAW 264.7 cells. RAW 264.7 cells were treated with 50 μg/mL of desulfated, deacetylated or desulfated–deacetylated CAF for 24 h. NO production was determined using Griess reaction. Data were means±SEM of three independent experiments. *Asterisks* show statistically significant difference from the native CAF treated group ($p<0.05$)

Consideration of endotoxin contamination

Endotoxin is known to stimulate macrophage and is often a contaminant in biological preparations. Therefore, to confirm that the CAF-induced NO production is not due to endotoxin contamination, CAF was tested for the presence of contaminating endotoxin using PMB, a pharmacological antagonist of endotoxin. As shown in Fig. 5, PMB dramatically decreased LPS-induced NO production in RAW 264.7 cells, but it did not affect CAF-induced NO production. The results demonstrated that CAF-induced NO production was not due to endotoxin contamination, but to CAF itself.

Involvement of sulfate and acetyl groups of CAF in CAF-induced NO production in RAW 264.7 cells

To confirm whether the involvement of sulfate and acetyl groups in CAF-induced NO production in RAW 264.7 cells, we evaluated the desulfated, deacetylated and desulfated–deacetylated CAF-induced NO production (Fig. 6). Desulfated and deacetylated CAF-induced NO production were low levels rather than native CAF. Desulfated–deacetylated CAF has little effect on NO production. The results indicated that sulfate and acetyl groups of CAF were important factors of CAF-induced NO production.

Involvement of TLR4, CD14 and SRA, but not CR3 in CAF-induced NO production in RAW 264.7 cells

To investigate the involvement of which membrane receptor in the NO production, we evaluated the effect of neutralizing anti-TLR4 (rat IgG_{2a}), anti-CD14 (rat IgG_{2b}), anti-CR3 (rat IgG_{2b}) and anti-SRA (rat IgG_{2b}) monoclonal antibodies in CAF-induced NO production in RAW 264.7 cells (Fig. 7). Rat normal IgG_{2a} and IgG_{2b} did not affect the CAF-induced NO production. On the other hand, neutralizing anti-TLR4, anti-CD14 and anti-SRA monoclonal antibodies significantly decreased CAF-induced NO production, but neutralizing anti-CR3 monoclonal antibody little affected. The results demonstrated that CAF-induced NO production was mediated through TLR4, CD14 and SRA.

Involvement of MAPK pathways in CAF-induced NO production

Several studies have reported that phosphorylation and activation of MAPK is critical in polysaccharide-induced

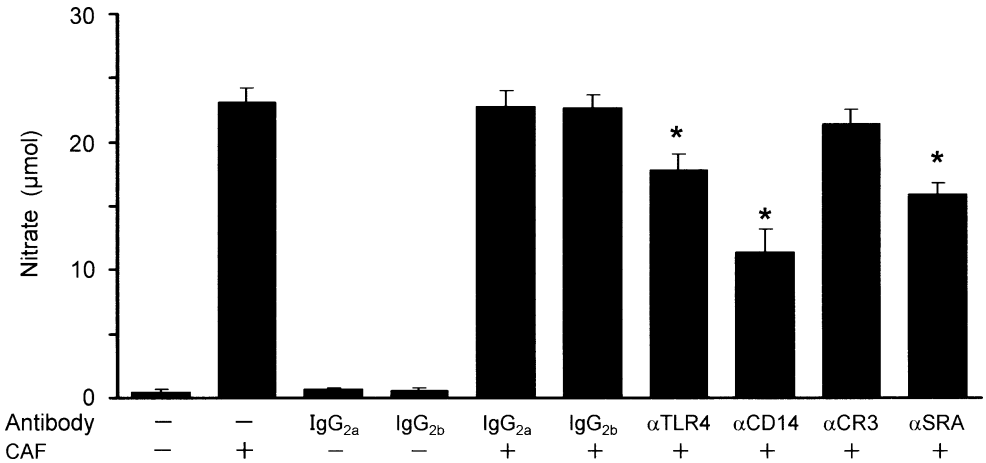

Fig. 7 Receptor mediated NO production induced in RAW 264.7 cells by CAF. RAW 264.7 cells were pretreated with neutralizing anti-TLR4 (αTLR4), anti-CD14 (αCD14), anti-CR3 (αCR3) or anti-SRA (αSRA) monoclonal antibodies (10 μg/mL) for 2 h, and then treated with CAF (50 μg/mL) for 24 h. The control groups were treated with rat normal IgG_{2a} or IgG_{2b} (10 μg/mL) for 2 h, and then treated with CAF (50 μg/mL) for 24 h. NO production was determined using Griess reaction. Data were means±SEM of three independent experiments. *Asterisks* show statistically significant difference from the IgG_{2a} or IgG_{2b} isotype-matched control groups ($p<0.05$)

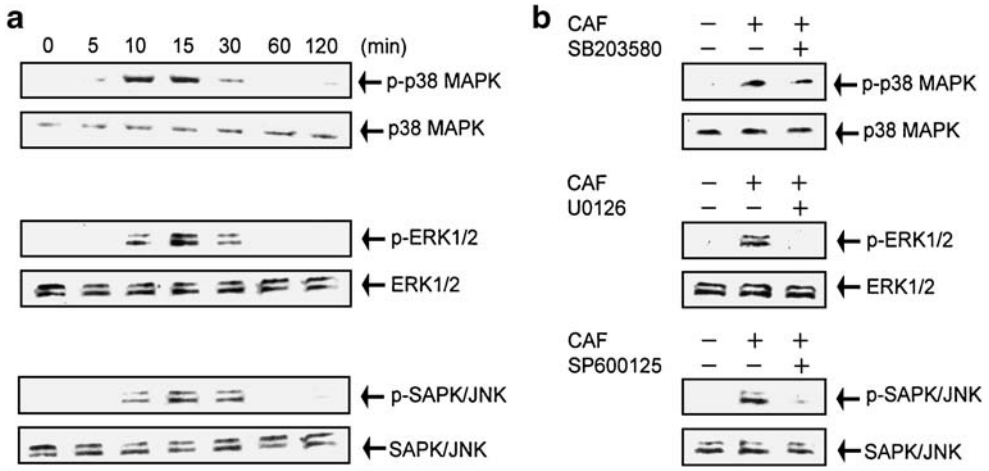

Fig. 8 **a** Time course of CAF-induced MAPKs phosphorylation in RAW 264.7 cells. RAW 264.7 cells were treated with CAF (50 μg/mL) for the indicated time. Cell lysates were prepared and subjected to SDS-PAGE and then immunoblot analysis for phospho-p38 MAPK, p38 MAPK, phospho-ERK1/2, ERK1/2, phospho-SAPK/JNK and SAPK/JNK. Phosphorylated p38 MAPK, ERK1/2 and SAPK/JNK is significantly detected in RAW 264.7 cells treated with CAF (50 μg/mL) for 10, 15 and 30 min. **b** Effect of MAPK inhibitors on CAF-induced MAPKs phosphorylation. RAW 264.7 cells were pretreated with SB203580, U0126 or SP600125 for 1 h before treatment with CAF (50 μg/mL) for 15 min. Cell lysates were prepared and subjected to SDS-PAGE and then immunoblot analysis for phospho-p38 MAPK, p38 MAPK, phospho- ERK1/2 and ERK1/2, phospho-SAPK/JNK and SAPK/JNK

NO production in macrophages [23–26, 35–37]. Therefore, to confirm weather CAF-induced NO production is mediated through the MAPKs activation, we monitored the levels of phosphorylated p38 MAPK, ERK1/2 and SAPK/JNK by immnoblot analysis, and evaluated the effect of specific MAPK inhibitors on CAF-induced NO production. Upon the treatment of RAW 264.7 cells with CAF (50 μg/mL), the levels of phosphorylated p38 MAPK, ERK1/2 and SAPK/JNK increased within 10 min and maintained after 30 min, and then decreased after 60 min (Fig. 8a). SB203580 (p38 MAPK inhibitor), U0126 (MEK1/2 inhibitor) and SP600125 (SAPK/JNK inhibitor) inhibited CAF-induced phosphorylation of p38 MAPK, ERK1/2 and SAPK/JNK, respectively (Fig. 8b). As shown in Fig. 9, SB203580 and SP600125 significantly inhibited CAF-induced NO production, while U0126 exhibited a little inhibition effect. Consequently, the results indicated that CAF induced activation of p38 MAPK, ERK1/2 and SAPK/JNK pathways, and the NO production was mediated mainly through p38 MAPK and SAPK/JNK pathways

Discussion

In this study, we investigated the chemical structural and immunological characteristics of CAF from commercially cultured *C. okamuranus*. The CAF consisted of α-1→3 linked L-fucosyl residues, where D-glucuronic acid is substituted at the C-2 and sulfate groups at the C-4. The acetyl groups also substituted at C-4 of the L-fucosyl residues. We proposed the chemical structure of CAF as illustrated in Fig. 2. The structural feature is similar to that proposed by some workers [4, 5], and corresponds as the rheological characteristics in aqueous solutions [6]

CAF induced activation of macrophages leading to NO, TNF-α and IL-6 production. Such effects were not due to endotoxin contamination, but to CAF itself. Consequently, CAF has potent effects on macrophage activation. CAF-induced NO production was significantly decreased by desulfation and deacetylation of CAF. The results suggested that sulfate and acetyl groups of CAF act as active sites, or

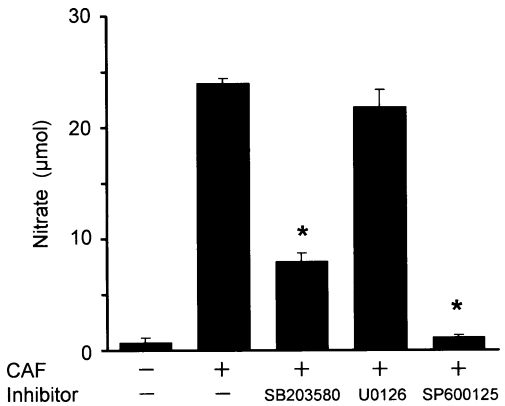

Fig. 9 Effect of MAPK inhibitors on CAF-induced NO production in RAW 264.7 cells. RAW 264.7 cells were pretreated with SB203580, U0126 or SP600125 for 1 h, and then treated with CAF (50 μg/mL) for 24 h. The NO production was determined using Griess reaction. Data were means±SEM of three independent experiments. *Asterisks* show statistically significant difference from the CAF treated group ($p<0.05$)

contribute to maintain the conformation of active form of CAF on CAF-induced NO production.

Macrophage activation by polysaccharides is mediated through pattern recognition receptors. The binding of polysaccharides to pattern recognition receptors induces several signal transduction events, which lead to macrophage activation. We found that CAF-induced NO production is mediated through TLR4, CD14 and SRA. It has been reported that several polysaccharides induced macrophage activation through TLR4, CD14 or SRA [23–27]. These suggested that TLR4, CD14 and SRA have wide specificity for polysaccharides. We also investigated that the role of MAPK, downstream signaling pathways of TLR4, CD14 and SRA signaling in a macrophage [43–46], on CAF-induced NO production. The results indicated that CAF induced p38 MAPK, ERK1/2 and SAPK/JNK activation, whereas CAF-induced NO production was mediated almost through activation of p38 MAPK and SAPK/JNK, but not ERK1/2. It has been reported that several polysaccharide-induced NO productions were mediated through p38 MAPK and SAPK/JNK but not ERK1/2, whereas cytokine production was mediated through p38 MAPK, ERK1/2 and SAPK/JNK [38]. Consequently, these evidences suggested that MAPK signaling pathways have critical roles in polysaccharide-induced macrophage activation and they individually regulate several macrophage responses. CAF-induced ERK1/2 activation may be involved in another kind of macrophage response. Our results suggested that CAF is recognized by membrane receptor TLR4, CD14 and SRA. These receptors lead to intracellular signaling cascades that converge to activate MAPK signaling pathways resulting in the NO production. That is to say, these are part of signaling pathways of CAF-induced macrophage activation.

In summary, we proposed a new primary structure of CAF. The CAF induced macrophage activation through membrane receptors TLR4, CD14 and SRA, and MAPK signaling pathways. The unique structural features of CAF may be of importance for its induction of macrophage activation and other biological activity. The present results suggested that CAF could be utilized as an immune stimulant.

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