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# Maillard-type glycated collagen with alginate oligosaccharide suppresses inflammation and oxidative stress by attenuating the expression of LPS receptors *Tlr4* and *Cd14* in macrophages†

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Inflammation and oxidative stress contribute to noncommunicable diseases (NCDs), with macrophages playing pivotal roles. Glycated collagen through Maillard-type glycation holds promise for enhancing anti-inflammatory properties, but its mechanism remains unclear. This study investigates the cellular mechanism and aims to contribute to expanding collagen utilization. Collagen was glycated with alginate oligosaccharide (AO) and glucose (Glc: as a comparative case) at 60 °C and 35% relative humidity for up to 24 h (C-AO and C-Glc, respectively). The anti-inflammatory activities of both C-AO and C-Glc were evaluated using an LPS-stimulated macrophage model. 18 h AO-glycated collagen (C-AO18 h) was found to significantly reduce the production of nitric oxide and proinflammatory cytokines (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β). In contrast, C-Glc did not exhibit enhanced anti-inflammatory activity during any of the glycation periods. The enhanced anti-inflammatory activity of C-AO18 h was attributed to its downregulating effect on LPS receptors (toll-like receptor 4, *Tlr4*; cluster of differentiation 14, *Cd14*) and myeloid differentiation primary response 88 (*Myd88*) mRNA expression, with suppression in receptor expression resulting in decreased phagocytic ability of macrophages against *E. coli*. In addition, compared with intact collagen, C-AO18 h exhibited improved antioxidant activity in the LPS-stimulated macrophage model, as it significantly upregulated superoxide dismutase (SOD) and catalase (CAT) activities while reducing malondialdehyde (MDA) levels. Overall, this study contributes to the development of collagen-based functional foods for mitigating inflammation and oxidative stress in NCDs.

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## 1. Introduction

Inflammation is a major defense response of the innate immune system. Excessive inflammation and oxidative stress can exacerbate noncommunicable diseases (NCDs) such as diabetes, cardiovascular disease, and respiratory disease, resulting in approximately 70% of all deaths.[1,2] Besides being a crucial mediator of inflammation, macrophages are key immune effector cells involved in both innate and acquired immune systems; they secrete various types of immune chemokines/cytokines and have phagocytic activity.[3] Studies have implicated macrophage activity in NCD development and progression. For example, in obesity-induced inflammation and NCDs, adipose tissue-secreted adipocytokines recruit macrophages, which then release proinflammatory mediators and further influence metabolic and inflammation pathways,[4–6] suggesting that controlling macrophage activity in the inflammation stage is necessary for effective NCD suppression.

Notably, the advancement of obesity instigates alterations in gut flora and diminished intestinal barrier function, leading to an increased concentration of blood endotoxins.[7] This, in turn, results in elevated blood endotoxin concentrations, intensifying chronic inflammation—a causative factor in the onset of NCDs. In other words, fluctuations in lipopolysaccharide (LPS) concentrations within the *in vivo* environment are implicated in the worsening of NCDs caused by the progression of chronic inflammation. LPS is a component of Gram-negative bacteria, and LPS-activated macrophages release various physiologically active substances, including nitric oxide (NO), tumor necrosis factor alpha (TNF-α), and interleukins (ILs), during inflammation.[8] LPS-stimulated RAW 264.7 macrophage-like cells are widely employed as an inflammation model to evaluate the anti-inflammatory function of

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food- and nonfood-derived products.[9] LPS-induced macrophage inflammation begins with the binding of LPS binding protein (LBP) to LPS, and cluster of differentiation 14 (CD14) then transfers LPS to the toll-like receptor 4 (TLR4)/myeloid differential protein 2(MD2) complex, which then activates the inflammation cascade through both the myeloid differentiation primary response 88 (MyD88)-dependent and MyD88-independent pathways.[10] Notably, under Gram-negative bacterial and LPS stimulation, the absence of TLR4 and CD14 leads to suppressed secretion of proinflammatory cytokines (*e.g.*, TNF-α and IL-6), resulting in reduced inflammation levels.[11,12] Therefore, both TLR4- and CD14-involving LPS reception is the crucial cellular component in LPS-induced inflammation.

As incorporating daily foods with strong anti-inflammatory and antioxidant properties is now widely known as an effective strategy to reduce inflammation levels and control NCDs,[13] enhancing the antioxidant and anti-inflammatory functions of food proteins has received increased attention. Specifically, the Maillard reaction is considered a promising approach to enhance protein functionalities, as it involves a spontaneous nonenzymatic reaction between basic amino acids (*e.g.*, lysine and arginine) and reducing sugars without requiring any chemical reagent.[14,15] Nishizawa *et al.*[16] and Li *et al.*[17] reported an enhanced anti-inflammatory function of salmon myofibrillar protein through this reaction. Although these research reports suggest that the Maillard reaction is an effective way to develop the anti-inflammatory activity of food proteins, which are beneficial ingredients for preventing NCDs, the enhanced anti-inflammatory activity of glycated collagen and its underlying mechanism remain unexplored. While we reported the enhanced cytoprotective activity of collagen against $H_2O_2$-induced cell oxidative stress through glycation with alginate oligosaccharide (AO) or glucose (Glc) in our previous study,[18] Cao *et al.*[19] indicated that $H_2O_2$ induced cell oxidative stress, further initiating the inflammation cascade. Therefore, the enhanced cytoprotective activity of glycated collagens against $H_2O_2$-induced cell oxidative stress suggests their potential anti-inflammatory function.

This study explores the improving effects of glycated collagen on anti-inflammatory and antioxidant activities using an LPS-stimulated macrophage model and elucidates the mechanism underlying its functional expression by quantifying intracellular signal transduction alterations. This study also examines several crucial factors in the immune system, including NO generation, proinflammatory cytokine secretion, LPS receptor mRNA expression, phagocytic activity, and endogenous antioxidant enzyme activities, to better comprehend the impact of glycated collagen on the immune response and oxidative stress.

## 2. Materials and methods

### 2.1. Materials

Tilapia scale collagen (acid soluble atelocollagen ATH-221, purity >99.99%, purchased from Koken Co., Ltd, Tokyo, Japan) and AO (mean degree of polymerization = 6, mean molecular mass: 1.1 kDa, prepared by alginate lyase degradation, and supplied by Hokkaido Mitsui Chemical Industry Co., Ltd, Hokkaido, Japan) were used in this study. Fujifilm Wako Pure Chemical Industries, Ltd (Osaka, Japan) supplied D(+)-glucose. Sigma Aldrich (St Louis, MO, USA) supplied porcine gastric mucosa-derived pepsin, bovine pancreas-derived trypsin, and *E. coli* (O111:B4)-derived LPS. The RAW 264.7 murine macrophage cell line was obtained from RIKEN, Institute of Physical and Chemical Research (Saitama, Japan). Thermo Fisher Scientific (Waltham, MA, USA) supplied Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin, while Nacalai Tesque (Kyoto, Japan) supplied nonessential amino acid solutions. Note that all other chemicals with no descriptions in the manuscript were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) or Wako Pure Chemical Industries Ltd (Osaka, Japan).

### 2.2. Reducing sugar attachment to collagen

Reducing sugars were attached to collagen as previously described.[18] Briefly, collagen was suspended in distilled water at a concentration of 2 mg mL$^{-1}$ and then mixed with AO or Glc at a weight ratio of 1 : 0.5. Subsequently, the mixture was homogenized using a Potter–Elvehjem homogenizer and freeze dried (FDU-2200, Tokyo Rikakikai, Co., Inc., Tokyo, Japan). The resulting freeze-dried mixture was incubated using a temperature–humidity cabinet (Sh-220, Tabai ESPEC Corp., Tokyo, Japan) under incubation conditions of 60 °C with 35% relative humidity for 0, 6, 12, 18, and 24 h to generate collagen-sugar conjugates (C-sugars). These produced C-sugars were referred to as C-AO (collagen glycated with AO) and C-Glc (collagen glycated with glucose). Intact collagen was also prepared as a control group (referred to as C). The obtained C-sugars were subjected to ethanol treatment to remove all unreacted sugars, as described in our previous study.[18] All experiments described below were performed using C-AO, C-Glc, and C, which contained no unreacted sugars.

### 2.3 Characterization of C-sugars

To characterize the glycated collagens prepared by the reaction for 0–24 h, the absorbance at 294 and 420 nm, available lysine content, and number of sugars attached to collagen were measured by the methods described in our previous study.[18] Since the glycated collagens (the reaction occurred for 0–18 h) analyzed in this study were the same samples as in the previous study,[18] some analytical data in Table 2 were treated as cited data.

### 2.4. Simulated gastrointestinal digestion of C-sugars *in vitro*

Stimulated gastrointestinal digestion was performed according to previous studies.[16–18] The glycated collagen was subjected to digestion using pepsin and trypsin to estimate its anti-inflammatory and antioxidant activities in LPS-stimulated RAW 264.7 cells. Briefly, several glycated and intact collagens at 2 mg mL$^{-1}$ concentrations were digested with pepsin (pH 2.0) and trypsin (pH 8.0) at 1% sample weight. The digestion was performed at 37 °C for 3 h for each enzyme at a digestion pH

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adjusted with 1 M HCl and 1 M NaOH, followed by 5 min boiling to terminate digestion. After 30 min of centrifugation at 20 000*g*, the supernatant was collected, lyophilized, and stored at −20 °C before use. All the following experiments were conducted using the digested C-sugars and C.

### 2.5. Cell culture

Raw 264.7 cells were cultured with DMEM containing 10% FBS, 100 units per mL penicillin, 0.1 mmol per L nonessential amino acids, and 100 μg per mL streptomycin at 37 °C under a 5% $CO_2$ humidified atmosphere. The cells were subcultured every 3 or 4 days and seeded in plates. After incubation with DMEM containing 10% FBS for 2 h, serum starvation was induced by replacing the culture medium with DMEM containing 0.1% FBS and further culturing for 24 h, followed by LPS and sample stimulation.

### 2.6. Cytotoxicity measurements

The cells were seeded into a 96-well plate at a density of $5 \times 10^3$ cells per well and cultured with DMEM containing 10% FBS for 2 h, and serum starvation was induced as described in section 2.5. Subsequently, the cells were treated with 500 μg $mL^{-1}$ of each digested C and C-sugar for 24 h. Cell viability/cytotoxicity was then measured using a CCK-8 assay kit (CCK-8, Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's protocol. The nontreated cells were set as a control group to evaluate the cell viability/cytotoxicity of each CP-sugar.

### 2.7. Quantification of TNF-α and IL-6

Raw 264.7 cells were cultured in a 96-well plate at a density of $2 \times 10^4$ cells per well with DMEM containing 10% FBS for 2 h, followed by serum starvation, as described in section 2.5. The cells were subsequently treated with digested C and C-sugars at 500 μg $mL^{-1}$ for 3 h, followed by co-stimulation with C or C-sugars and LPS for 24 h at a final concentration of 500 μg $mL^{-1}$ and 0.2 μg $mL^{-1}$, respectively. The culture supernatant was collected *via* centrifugation to quantify TNF-α and IL-6 levels. Quantification was performed using an ELISA kit (BioLegend, San Diego, CA, USA) following the manufacturer's protocol.

### 2.8. Quantification of IL-1β and NO

The treatment of cells for IL-1β and NO quantification followed the protocol described in section 2.7, with slight modifications. In addition, according to Xu *et al.*,[20] the cells were seeded at a density of $2 \times 10^5$ cells per well in a 96-well plate, and the LPS concentration used was 1 μg $mL^{-1}$. After collecting the culture supernatant by centrifugation, the IL-1β level was measured using an ELISA kit (BioLegend, San Diego, CA, USA.) following the manufacturer's protocol. The Griess method with the Griess reagent (Promega, WI, USA) was employed to evaluate the NO content.[21]

### 2.9. RNA isolation and quantitative reverse transcription–polymerase chain reaction

The Raw 264.7 cells were seeded in a 3 cm dish at a density of $5 \times 10^5$ cells per dish, followed by serum starvation as described in section 2.5. After treating the cells with digested C-sugars at 500 μg $mL^{-1}$ for 3 h, LPS was loaded to each plate and the cells were co-stimulated with C-sugars and LPS for 6 h at a final concentration of 500 μg $mL^{-1}$ and 0.2 μg $mL^{-1}$, respectively. For LPS-stimulation-free cells, only C-sugar treatment was performed at a concentration of 500 μg $mL^{-1}$ for 24 h. Finally, after removing the culture medium and washing with PBS, RNA extraction was performed.

RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality and concentration were measured using a NanoDrop (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). After converting 1 μg RNA to cDNA using the ReverTra Ace® qPCR RT Master Mix with a gDNA Remover (Toyobo Co., Ltd Osaka, Japan), the Taqman® gene expression assay was conducted using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) to evaluate the mRNA expression of *Tnfa*, *Il6*, *Il1b*, *Tlr4*, *Cd14*, and *Myd88*. Ribosomal protein, large, P0 (*Rplp0*) was employed as the housekeeping gene. Table 1 contains all the primers and probes used.

### 2.10. Phagocytic activity

After the serum starvation described in section 2.5, the Raw 264.7 cells ($5 \times 10^4$ cells per well) were treated with digested C-sugars at 500 μg $mL^{-1}$ or cytochalasin D at 0.2 μM for 24 h, followed by culturing with enzyme-labeled *E. coli* (supplied by the kit) for 6 h. Cytochalasin D was employed as a negative control as an inhibitor of phagocyte activity in the present study. The effect of C-sugars on phagocytosis was measured using a CytoSelect 96-well phagocytosis assay kit (#CBA-222, Cell Biolabs, Inc., San Diego, CA, USA) following the manufacturer's protocol.

**Table 1** The primer sequences for quantitative reverse transcription–polymerase chain reaction

| Name | Forward primer (5′–3′) | Reverse primer (3′–5′) | TaqMan assay ID | NCBI reference sequences |
|---|---|---|---|---|
| *Rplp0* | CAGGATGAAAAAGCAAAACCGC | TGGCACCAAGTCAAGAGACTG | Mm00725448_s1 | NM_007475.5 |
| *Tnfa* | ACTGAACTTCGGGGTGATCG | AGGGTCTGGGCCATAGAACT | Mm00443258_m1 | NM_013693.3 |
| *Il6* | GACTGATGCTGGTGACAACC | CTGTGACTCCAGCTTATTGTT | Mm00446190_m1 | NM_031168.2 |
| *Il1b* | GCTCATCTGGGATCCTCTCC | CAGCTTCTCCACAGCCACAA | Mm00434228_m1 | NM_008361.4 |
| *Tlr4* | GTCAGCAAACGCCTTCTTCC | TGCTGAGTTTCTGATCCATGC | Mm00445273_m1 | NM_021297.3 |
| *Cd14* | ATCCTCCTGGCACAGAATGC | GTACAATTCCACATCTGCCGC | Mm00438094_g1 | NM_009841.4 |
| *Myd88* | AGAGCTGCTGGCCTTGTTAG | GACTCCTGGTTCTGCTGCTT | Mm00440338_m1 | NM_010851.3 |

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### 2.11. Quantification of superoxide dismutase and catalase activities and malondialdehyde content

Cells were cultured in 6 cm dishes at a density of $2 \times 10^6$ cells per dish. After serum starvation treatment, as described in section 2.5, the cells were cultured with 500 μg $mL^{-1}$ digested C-sugars for 3 h and were then co-stimulated with C-sugars and LPS for 24 h at a final concentration of 500 μg $mL^{-1}$ and 1 μg $mL^{-1}$, respectively. After washing with PBS, the cells were collected, lysed by sonication, and homogenized. After centrifuging the samples at 10 000*g* for 30 min at 4 °C, the supernatants were employed for each measurement. Each supernatant protein concentration was determined using a Protein Assay Rapid Kit (Fujifilm Wako Pure Chemical Co., Tokyo, Japan).

The SOD and CAT activities of each cell lysate were assessed using an SOD Assay Kit-WST (#S311, Dojindo Laboratories, Kumamoto, Japan) and a Catalase Colorimetric Activity Kit (#K033-H1, Arbor Assay, Ann Arbor, MI, USA), respectively. The SOD and CAT activity results were normalized to the protein concentration and expressed as U $mg^{-1}$ protein. The MDA content in each cell lysate was measured using an MDA Assay Kit (#M496, Dojindo Laboratories), and the results were normalized to the protein concentration and expressed as nmol $mg^{-1}$ protein.

### 2.12. Statistical analysis

Data were subjected to Tukey–Kramer's test using JMP software (version 16, SAS Institute Inc., Cary, NC, USA), $P < 0.05$ was considered statistically significant. All results were expressed as mean ± SD.

## 3. Results and discussion

### 3.1. Characterization of collagen–sugar conjugates

Table 2 lists the absorbance, available lysine content, and the number of conjugated reducing sugars of all glycated collagens. Because the relationships between the three Maillard reaction indicators of AO- or Glc-glycated collagens up to 18 h have been discussed in previous studies,[18] Table 2 lists the profile results of the 24-h glycated collagens. The absorbance at 294 and 420 nm (A294 and A420, respectively), and the number of conjugated sugars of the C-AO group still increased upon a 24 h extension in the reaction time, while the available lysine content slightly decreased. However, the C-Glc group showed a different pattern in each Maillard reaction indicator because both A294 and A420 were reduced after a 24 h reaction, with the available lysine content continuously decreasing. In addition, the numbers of conjugated sugars slightly increased, significantly exceeding those of the C-AO group. These results indicate faster glycation in C-Glc than in C-AO. Notably, the decrease in ultraviolet absorption components and slowing down of the browning reaction observed when the Maillard reaction progresses to the advanced stage could explain the decreased A294 and A420 values of C-Glc during glycation up to 24 h.[22,23] As seen in Table 2, the Maillard reac-

**Table 2** Characterization of collagen glycation products

| Reaction time (h) | A294 | | | A420 | | | Available lysine content[b] (%) | | | Reducing sugar conjugation (nmol per mg protein) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | C-AO | C-Glc | C | C-AO | C-Glc | C | C-AO | C-Glc | C | C-AO | C-Glc |
| 0[a] | 0.40 ± 0.04[a] | 0.41 ± 0.01[a] | 0.46 ± 0.02[a] | 0.18 ± 0.02[a] | 0.19 ± 0.01[a] | 0.24 ± 0.01[a] | 100.00 ± 5.76[a] | 96.47 ± 1.73[a] | 100.62 ± 1.39[a] | — | 7.96 ± 0.99[a] | 9.94 ± 1.54[a] |
| 6[a] | 0.41 ± 0.03[a] | 0.76 ± 0.02[b] | 1.28 ± 0.01[b] | 0.18 ± 0.03[a] | 0.36 ± 0.03[b] | 0.84 ± 0.01[b] | 97.88 ± 6.53[a] | 91.27 ± 0.45[b] | 64.47 ± 1.76[b] | — | 39.64 ± 2.11[b] | 106.15 ± 3.04[b] |
| 12[a] | 0.41 ± 0.1[a] | 0.81 ± 0.02[c] | 1.32 ± 0.01[c] | 0.18 ± 0.01[a] | 0.36 ± 0.02[b] | 0.90 ± 0.02[c] | 98.66 ± 2.65[a] | 89.84 ± 2.25[bc] | 57.60 ± 3.34[c] | — | 47.06 ± 2.55[c] | 150.06 ± 6.00[c] |
| 18[a] | 0.43 ± 0.01[a] | 1.06 ± 0.01[d] | 1.36 ± 0.01[d] | 0.18 ± 0.04[a] | 0.47 ± 0.01[c] | 0.91 ± 0.01[c] | 97.93 ± 8.0[a] | 85.57 ± 2.72[cd] | 38.06 ± 0.85[d] | — | 59.32 ± 3.44[d] | 256.62 ± 1.65[d] |
| 24 | 0.41 ± 0.02[a] | 1.36 ± 0.02[e] | 1.29 ± 0.02[bc] | 0.18 ± 0.02[a] | 0.61 ± 0.03[d] | 0.86 ± 0.02[bc] | 96.61 ± 4.46[a] | 82.36 ± 1.37[d] | 34.54 ± 2.42[e] | — | 66.12 ± 3.75[e] | 262.70 ± 8.67[d] |

[a] Data from 0 to 18 h were quoted from our previous study, which was duplicated for one time.[18] [b] The available lysine content was presented as the relative percentage of C reacted for 0 h. A294: absorbance at 294 nm; A420: absorbance at 420 nm; C: collagen; C-AO: collagen glycated with AO; C-Glc: collagen glycated with glucose. The results are expressed as the mean ± SD ($n$ = 3), and bars with different letters indicate significant differences ($P < 0.05$, by Tukey–Kramer multiple comparison test).



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tion of the C-Glc group has already progressed to the advanced stage within 24-h glycation, with that of the C-AO group staggering behind.

### 3.2. Effect of C-sugars on cell viability/proliferation and NO generation

The effect of C-sugars on the viability/proliferation of macrophages was assessed (Fig. 1A and B). The fact that no decreased cell viability was observed in all C and C-sugar-treated cells indicated that C and C-sugars exhibited no cytotoxic activity. Fig. 1C and D show the effect of C-AO and C-Glc on NO generation in LPS-stimulated murine macrophage cells, respectively. LPS treatment of macrophages significantly enhanced the NO production level compared to the control group. However, C-AO inhibited NO production in LPS-stimulated macrophages, which increased as the reaction time progressed. C-AO after 18 h of glycation (C-AO18 h) induced the most effective inhibitory activity on NO production, reaching 71% of the LPS group and maintaining up to 24 h of glycation (approximately 74% of the LPS group, Fig. 1C). In contrast, C-Glc treatment in all glycation periods had no reducing effect on the elevated levels of NO in LPS-stimulated macrophages (Fig. 1D).

NO is a bioactive molecule associated with bacterial infection and inflammation. LPS-activated macrophages generate excessive NO, which can lead to oxidative stress in cells, reduced intracellular antioxidant enzyme activities, and lipid peroxidation.[23] Thus, suppressing NO expression during the inflammation stage is indicative of anti-inflammatory and antioxidant activities. As seen in Fig. 1C and D, AO glycation effectively improved the anti-inflammatory and antioxidant activity of collagens on LPS-stimulated macrophages.

### 3.3. Effect of C-sugars on the secretion of proinflammatory cytokines

To profile the anti-inflammatory activity of C-sugars, the production of TNF-α, IL-6, and IL-1β was evaluated in LPS-stimulated macrophages following C-sugar treatment.

Fig. 2A shows the suppressive effect of C-AO on the secretion of TNF-α, IL-6, and IL-1β in LPS-stimulated macrophages. LPS stimulation considerably increased the TNF-α, IL-6, and IL-1β levels compared to the control group. C suppressed the production of these cytokines, which was at the same level as that of 0-h glycated C-AO (C-AO0 h), indicating the anti-inflammatory activity of intact collagen digestion. The inhibitory activity of C-AO on TNF-α, IL-6, and IL-1β production

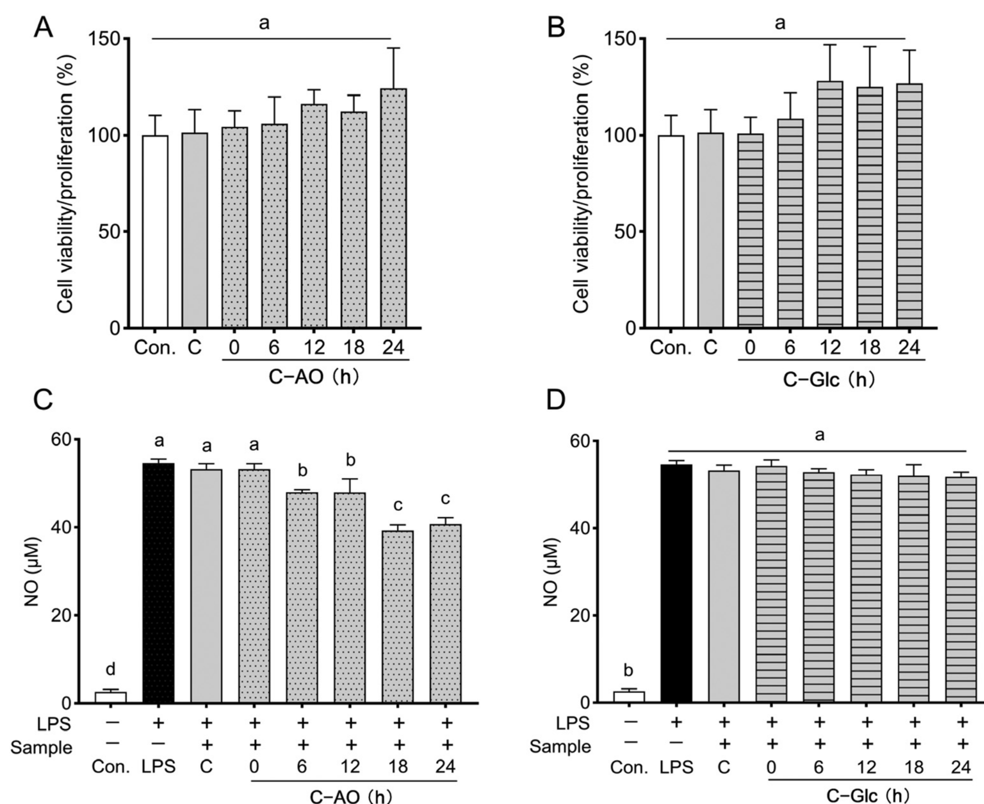


Fig. 1 Effect of C-sugars on viability/proliferation and NO production of macrophages. Cell viability/proliferation of C-AO (A) and C-Glc (B); Con.: control group, representing the cells without C-sugar treatment; C: intact collagen digest-treated cells. Effect of C-AO (C) and C-Glc (D) on NO production in LPS-stimulated macrophages; Con.: control group, representing the cells without LPS or C-sugar treatment; LPS: LPS group, representing the cells treated with only LPS; C: intact collagen digest group, representing the cells receiving both LPS and collagen digest treatment. The results are expressed as the mean ± SD ($n$ = 5), and bars with different letters indicate significant differences ($P$ < 0.05, by the Tukey–Kramer multiple comparison tests).

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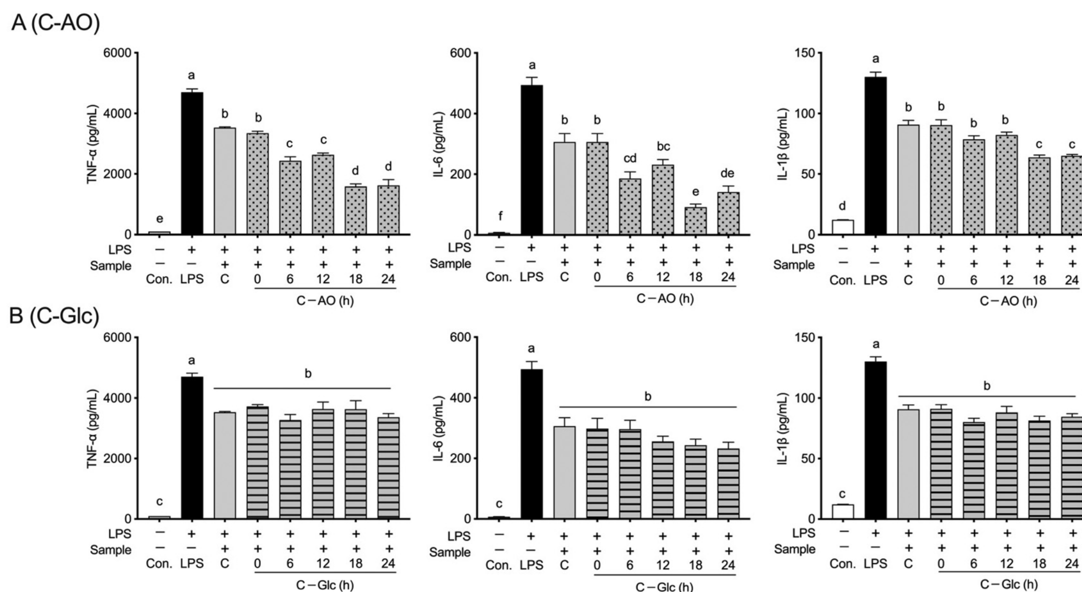


Fig. 2 Effect of C-sugars on the secretion of TNF-α, IL-6, and IL-1β. Effect of C-AO (A) and C-Glc (B) on the secretion of TNF-α, IL-6, and IL-1β in LPS-stimulated macrophages. Con.: control group, representing the cells without LPS or C-sugar treatment. LPS: LPS group, in which the cells were treated with LPS only. C: intact collagen digest group, in which the cells were treated with LPS and collagen digests. The results are expressed as the mean ± SD ($n$ = 5), and bars with different letters indicate significant differences ($P$ < 0.05, by the Tukey–Kramer multiple comparison test).

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was enhanced as the Maillard reaction progressed, similar to the improved inhibition observed for NO production. The most effective inhibitory activity on the production of the three cytokines was confirmed at 18 h glycation (C-AO18 h; TNF-α: approximately 33%, IL-6: approximately 18%, and IL-1β: approximately 50%, relative to the LPS group). Notably, the significantly enhanced anti-inflammatory activity of C-AO18 h was maintained until 24 h of glycation.

Fig. 2B shows that the inhibitory activity of 0-h glycated C-Glc (C-Glc0 h) on cytokine production is the same as that of C and C-AO0 h, suggesting that intact collagen digestion contributes to the anti-inflammatory activity of C-Glc0 h. Unlike the C-AO group, the C-Glc group exhibited no significant improvement in inhibitory activity during the glycation process, except for IL-6 production, which continuously decreased after 24 h glycation.

Overall, collagen digestion exhibited anti-inflammatory activity, contributing to the functionality of both C-AO0 h and C-Glu0 h. However, while AO glycation enhanced the anti-inflammatory activity of collagen, Glc glycation exhibited no alterations. As the reducing sugars employed in this study have no anti-inflammatory activity (ESI Fig. 1†), the enhanced anti-inflammatory activity of C-AO is due to Maillard-type glycation rather than the direct involvement of individual reducing sugars.

### 3.4. Suppression of inflammation-related gene expression by C-AO treatment

To clarify the mechanism by which AO glycation enhances the anti-inflammatory activity of collagen, the effects of the C-AO18 h sample, which showed the most effective anti-inflammatory activity, on the expression of inflammation-related genes were examined in LPS-stimulated macrophages. The C-Glc18 h sample was also employed in this test. Because the C sample and 0 h glycated C-sugar samples had the same anti-inflammatory activity level (Fig. 1 and 2), the 0 h glycated C-sugars (C-AO0 h and C-Glc0 h) were utilized to evaluate the enhanced anti-inflammatory function of the 18 h glycated C-sugars.

Fig. 3A–C depicts the effect of C-sugars on *Tnfa*, *Il6*, and *Il1b* mRNA expressions in LPS-treated macrophages. Both C-AO0 h and C-Glc0 h slightly reduced the TNF-α, IL-6, and IL-1β mRNA expression levels, which was attributed to the intact collagen digestion observed in Fig. 2. Glycation with AO for 18 h (C-AO18 h) improved collagen inhibition activity in the mRNA expression of proinflammatory cytokines, whereas no significant difference was observed in C-Glc18 h. These results indicate that intact collagen can suppress the transcription of proinflammatory cytokines, with AO glycation for 18 h enhancing suppression activity.

LPS receptors, such as TLR4 and CD14, are crucial components in LPS-induced macrophage inflammation. Synergizing LPS with TLR4 and CD14 can trigger the inflammation cascade, which is transduced through the TLR4-MyD88 signaling pathway, resulting in the secretion of proinflammatory cytokines.[10] The significant role of LPS receptors and MyD88 in response to LPS induction has already been demonstrated in animal models. Hoshino *et al.*[24] demonstrated that macrophages from TLR4-deficient mice did not respond to LPS induction, and other researchers did not observe LPS response in CD14 or MyD88 knockout mice.[25,26] Xia *et al.*[27] also reported that the suppressed inflammation level in LPS-

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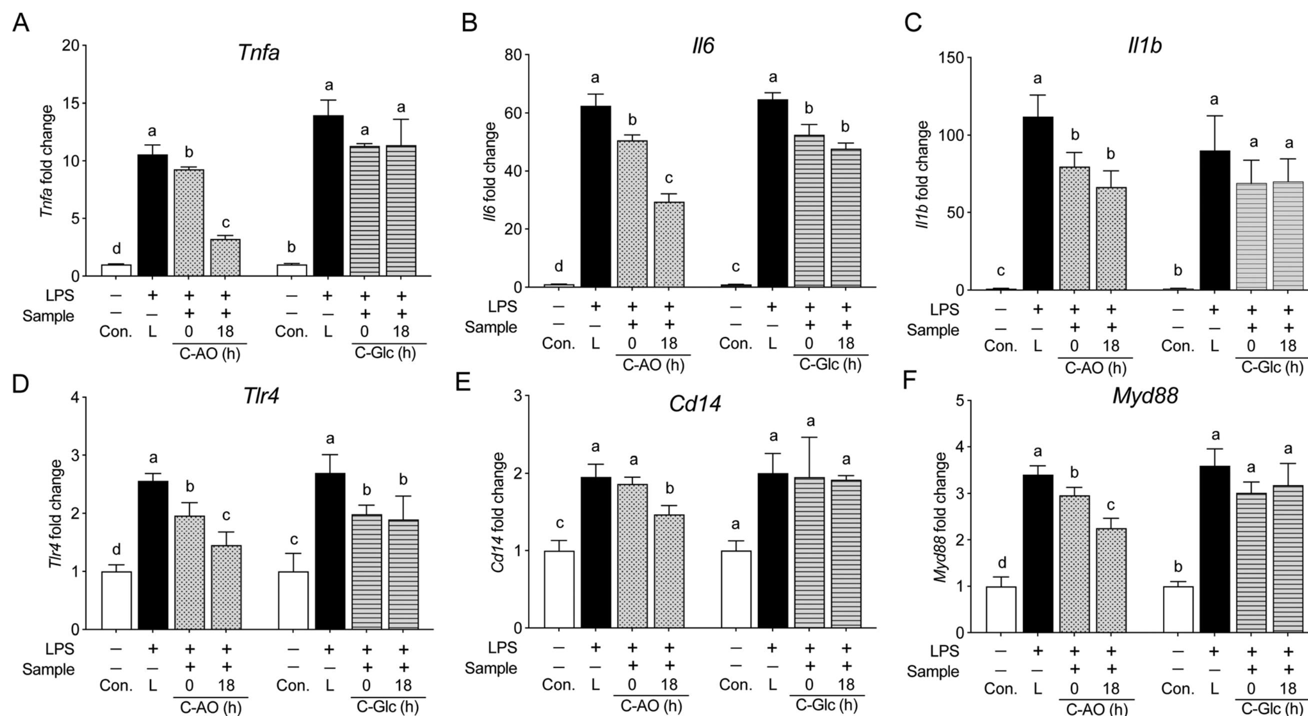


**Fig. 3** Effect of C-sugars on LPS receptor expression in LPS-stimulated macrophages. Effect of C-sugars on mRNA expression in LPS-stimulated macrophages of *Tnfa* (A), *Il6* (B), *Il1b* (C), *Tlr4* (D), *Cd14* (E), and *Myd88* (F). Con.: control group, in which the cells were not treated with C-sugars and LPS; LPS: LPS group, in which the cells received only LPS treatment. The results are expressed as the mean ± SD ($n$ = 5), and bars with different letters indicate significant differences ($P$ < 0.05, by the Tukey–Kramer multiple comparison test).

stimulated macrophages was attributed to reduced *Tlr4*, *Cd14*, and *Myd88* mRNA expression levels. For collagen-related ingredients, Xing *et al.*[28] found that the inhibitory activity of bone gelatin peptides in the expression/production of TLR4 at the mRNA/protein levels led to anti-inflammatory activity in the inflammatory bowel disease of mice. Therefore, in this study, the suppressive effects of C-AO0 h and C-AO18 h on *Tlr4*, *Cd14*, and *Myd88* mRNA expression were examined in LPS-stimulated and non-LPS-stimulated macrophages.

Fig. 3D–F shows that LPS stimulation increased LPS receptors (*Tlr4* and *Cd14*) and their downstream *Myd88* mRNA expression. C-AO0 h and C-Glc0 h treatment in response to LPS induction reduced *Tlr4* and *Myd88* mRNA expressions compared to the LPS group, whereas no significant effect was observed in *Cd14* mRNA expression. However, simultaneous repression in *Tlr4*, *Cd14*, and *Myd88* expression occurred when LPS-stimulated macrophages underwent C-AO18 h treatment. As observed in the expression of cytokines, C-Glc18 h exhibited no enhanced suppressive function in *Tlr4*, *Cd14*, and *Myd88* expressions compared to C-Glc0 h. In addition, the suppressive effects of C-AO18 h on *Tlr4* and *Cd14* mRNA expression were also confirmed in non-LPS-stimulated macrophages (Fig. 4). These results indicate that collagen glycated with AO for 18 h suppressed inflammation by regulating the mRNA expressions of LPS receptors *Tlr4*, *Cd14*, and their downstream *Myd88*.

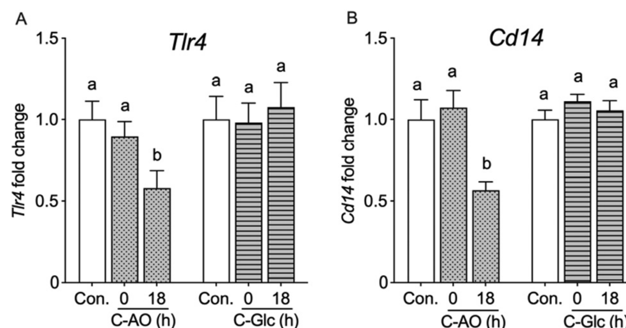


**Fig. 4** Effect of C-sugars on the LPS receptor expression of macrophages without LPS stimulation. Effect of C-AO on the mRNA expression of *Tlr4* (A) and *Cd14* (B) in non-LPS-stimulated macrophages; Con.: control group, in which the cells were not treated with C-sugars. The results are expressed as the mean ± SD ($n$ = 5), and bars with different letters indicate significant differences ($P$ < 0.05, by the Tukey–Kramer multiple comparison test).

### 3.5. Suppressive effect of C-AO on the phagocytosis of *E. coli*-stimulated macrophages

Phagocytosis is an essential function of macrophages related to the defense against invading pathogens, and it is the first step in triggering host defense and inflammation.[29] Activated TLR4 upregulated the phagocytosis of macrophages through MyD88.[29,30] Anand *et al.*[31] observed that LPS stimulation sig-

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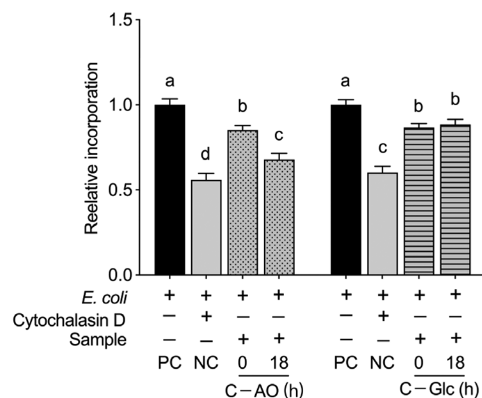


Fig. 5 Effect of C-sugars on the phagocytic activity of macrophages. PC: positive control, in which the cells were only treated with *E. coli*; NC: cytochalasin D-treatment group, in which the cells were treated with both cytochalasin D and *E. coli*. The results are expressed as the mean ± SD ($n$ = 4), and bars with different letters indicate significant differences ($P$ < 0.05, by Tukey–Kramer multiple comparison test).

nificantly enhanced the phagocytic activity of peritoneal macrophages harvested from wild-type mice but did not enhance that of macrophages harvested from TLR4-mutant mice. As these studies indicate a proportional relationship between TLR4 and phagocytosis, suggesting that phagocytic activity is essential for the inflammatory responses of macrophages, we examined the phagocytic activity of C-sugar-treated macrophages.

As shown in Fig. 5, macrophages stimulated by *E. coli* alone (positive control group) exhibited significantly enhanced phagocytic activity, whereas cytochalasin D treatment (negative control group) reduced phagocytosis compared to the positive control group. In contrast, macrophages treated with C-AO0 h and C-Glc0 h demonstrated a slight reduction in phagocytic activity. Interestingly, C-AO18 h treatment significantly inhibited the phagocytic activity of macrophages, whereas C-Glc18 h exhibited no reduction effect compared to the C-Glc0 h-treated cells.

The modulating function of Maillard reaction products on the phagocytic activity of macrophages has been reported. Treatment with lactose-glycated caseinate and glycation products generated from bovine serum albumin inhibited the phagocytic activity of macrophages.[3,32] In the present study, Fig. 3D–F and 4 show that AO-glycated collagen treatment suppressed the expression of *Tlr4*, a receptor for Gram-negative bacteria, in macrophages. Therefore, the suppression of phagocytosis by the Maillard reaction products, including C-AO18 h, may be associated with the downregulation of *Tlr4* and *Myd88* mRNA expression.

### 3.6. C-AO treatment suppressed LPS-induced oxidative stress in macrophages

Reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as superoxide and NO, are produced by macrophages in response to LPS stimulation.[33] The generated ROS and RNS could damage bioactive molecules such as protein and DNA while activating nuclear factors such as nuclear factor-kappa B (NF-κB) to exacerbate inflammation.[34,35] As collagen's inhibitory activity on NO production in LPS-stimulated macrophages was enhanced by glycation, suggesting enhanced antioxidant activity of C-sugars, we investigated the effects of these C-sugars on the MDA content and SOD and CAT activities in LPS-activated macrophages (Fig. 6).

LPS stimulation significantly increased MDA levels in macrophages, suggesting inflammation-induced oxidative damage (Fig. 6A). However, the C-AO0 h-treated macrophages exhibited a reduced MDA level, and the C-Glc0 h-treated macrophages demonstrated a slightly reduced MDA level, although not significant. Treating macrophages with C-AO18 h

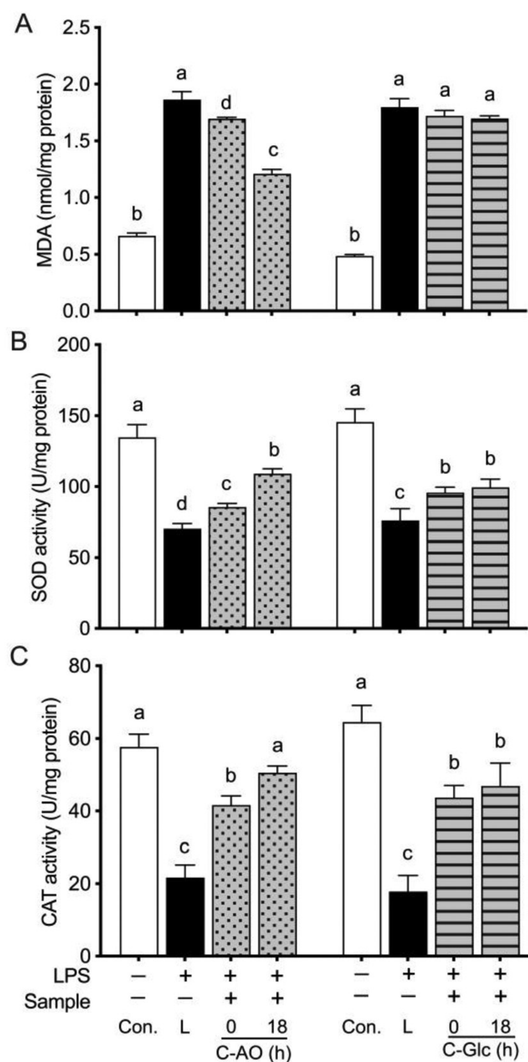


Fig. 6 Effect of C-AO on SOD and CAT activity and MDA content in LPS-stimulated macrophages. (A) MDA content. (B) SOD activity. (C) CAT activity. Con.: control group, in which the cells were not treated with C-sugars and LPS; L: LPS group, in which the cells were treated only with LPS. The results are expressed as the mean ± SD ($n$ = 3), and bars with different letters indicate significant differences ($P$ < 0.05, by the Tukey–Kramer multiple comparison test).

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further reduced the MDA level, but no effect was observed in the C-Glc18 h-treated cells. MDA, a product generated from lipid peroxidation, is widely used to reflect cell damage from reactive oxygen metabolites.[36] Collagen protected macrophages from oxidative damage during inflammation stages, and AO glycation improved this protective activity (Fig. 6A).

Fig. 6B and C illustrate the effect of C-sugars on SOD and CAT activities in LPS-stimulated macrophages, respectively. SOD and CAT activities were reduced in LPS-stimulated macrophages. Nevertheless, the reduced activity of the antioxidant enzymes was partially recovered after C-AO0 h and C-Glc0 h treatment. Likewise, C-AO18 h exhibited a more practical function in enhancing the activity of antioxidant enzymes, but C-Glc18 h did not exhibit a significant enhancement in activity.

SOD and CAT are two essential antioxidant enzymes that modulate cellular oxidative stress. Despite the complexity of oxidative stress–inflammation relationships, the activity of antioxidant enzymes such as SOD and CAT are reduced in the inflammatory cells, which then breaks redox homeostasis and induces cell death.[37–39] Various studies have reported that the Maillard reaction products could enhance SOD and CAT activities during inflammation stages, which could contribute to the anti-inflammatory functions and protect the model animals from oxidative/inflammation damage.[40,41] Thus, the results presented in Fig. 6 indicate that collagen could suppress the LPS-induced oxidative stress in cells by enhancing the antioxidant enzyme activities and reducing the MDA level, with C-AO18 h promoting the protective function of collagen against LPS-induced oxidative stress.

Recent works reveal that oxidants and oxidative stress affect the release of endogenous danger signal molecules from damaged tissues, including their sensing by the toll-like (TLR) and NOD-like (NLR) families of innate immune receptors, and as a result, oxidative stress activates signaling pathways that initiate cellular responses, such as induction of inflammation.[42,43] Considering these findings, it is likely that the suppressive effect of C-sugar on the secretion of inflammatory mediators from macrophages is not only attributed to its intrinsic anti-inflammatory properties but also influenced by its antioxidant capacity, particularly its potential to inhibit oxidative stress.

Overall, this study demonstrated that AO glycation enhances the antioxidant activity of fish collagen in LPS-induced cell oxidative stress and contributes to the improved anti-inflammatory activity of glycated collagen. In this study, we investigated glycated collagen peptides derived from pepsin-trypsin digestion, assuming behavior similar to that *in vivo*. When ingested, glycated collagen undergoes digestion and absorption, potentially influencing macrophages as peptides. Therefore, experiments were conducted using pepsin-trypsin digests to investigate the effects of glycated collagen under conditions mimicking those *in vivo*. Considering the structure of glycated collagen, it is estimated that both the generation of novel functional peptides arising from changes in digestion patterns due to glycation and Maillard-type glycated peptides contribute to its antioxidant and anti-inflammatory functions. In addition, a recent study by Li *et al.* (2023)[17] highlighted the significance of carboxy groups present in reducing sugars for enhancing the anti-inflammatory function of the Maillard-type glycated fish proteins. Therefore, it is essential to investigate the involvement of both factors—novel functional peptides and glycosyl units—to elucidate the factors contributing to improved antioxidant and anti-inflammatory activities.

## 4. Conclusion

C-AO18 h demonstrated improved anti-inflammatory activity in LPS-stimulated macrophages. This effect was attributed to downregulated LPS receptor mRNA expression, resulting in the suppression of proinflammatory cytokines and NO secretion. C-AO18 h also exhibited antioxidant activity in an inflammatory environment by boosting the activity of antioxidant enzymes such as SOD and CAT while reducing MDA content. Overall, the notable anti-inflammatory and antioxidant effects of C-AO on macrophages hold promise for NCD prevention, expanding the potential applications of collagen as a beneficial component in both foods and materials.

## Abbreviations

| | |
|---|---|
| A294 | Absorbance at 294 nm |
| A420 | Absorbance at 420 nm |
| AO | Alginate oligosaccharide |
| C | Collagen |
| C-AO | Collagen glycated with alginate oligosaccharide |
| C-Glc | Collagen glycated with glucose |
| CAT | Catalase |
| CD14/Cd14 | Cluster of differentiation 14 (protein/mRNA) |
| DMEM | Dulbecco's modified Eagle's medium |
| FBS | Fetal bovine serum |
| Glc | Glucose |
| IL-6/Il6 | Interleukin 6 (protein/mRNA) |
| IL-1β/Il1b | Interleukin 1 beta (protein/mRNA) |
| LBP | Lipopolysaccharide-binding protein |
| LPS | Lipopolysaccharide |
| MD2 | Myeloid differential protein 2 |
| MDA | Malondialdehyde |
| MyD88/Myd88 | Myeloid differentiation primary response 88 (protein/mRNA) |
| NCDs | Noncommunicable diseases |
| NF-κB | Nuclear factor-kappa B |
| NO | Nitric oxide |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RPLP0 | Ribosomal protein, large, P0 |
| SOD | Superoxide dismutase |
| TNF-α/Tnfa | Tumor necrosis factor alpha (protein/mRNA) |
| TLR4/Tlr4 | Toll-like receptor 4 (protein/mRNA) |

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## Author contributions

Boxue Yang: conceptualization, validation, investigation, and writing – original draft. Wenzhao Li: investigation and resources. Hiroki Saeki: conceptualization, resources, and writing – review & editing. Yutaka Shimizu: resources. Ga-Hyun Joe: conceptualization, resources, and writing – review & editing.

## Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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