Effects of Sulfated Tremella Polysaccharide and Codonopsis pilosula Polysaccharide on Chicken T Lymphocyte Proliferation and Interleukin-2 mRNA Expression Level: Postprint

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Abstract

To investigate the immunoenhancement mechanisms of sulfated Tremella polysaccharide (sTPS70c) and sulfated Codonopsis polysaccharide (sCPPS50c), this study used unmodified Tremella polysaccharide (TPStp) as a control and employed the thiazolyl blue (MTT) assay and real-time fluorescence quantitative PCR to determine the effects of sTPS70c and sCPPS50c on chicken T lymphocyte proliferation and interleukin-2 (IL-2) mRNA expression levels. The results showed that when polysaccharides were added alone to peripheral blood lymphocytes, sTPS70c and sCPPS50c at almost all concentrations could significantly stimulate T lymphocyte proliferation ($P<0.05$), whereas TPStp only significantly stimulated T lymphocyte proliferation at a concentration of 3.125 g/mL ($P<0.05$); when polysaccharides and phytohemagglutinin P (PHA-P) were simultaneously added to peripheral blood lymphocytes, sCPPS50c significantly stimulated lymphocyte proliferation at concentrations of 0.391~1.563 g/mL ($P<0.05$); sTPS70c and sCPPS50c at a concentration of 1.563 g/mL could enhance IL-2 mRNA expression in T lymphocytes ($P<0.05$), with sTPS70c exhibiting a significantly stronger promoting effect on IL-2 mRNA expression than unmodified TPStp ($P<0.05$), and this effect was strongest at the concentration of 1.563 g/mL. These results suggest that sulfation modification can enhance the lymphocyte proliferation activity of polysaccharides and significantly increase IL-2 mRNA expression, with sTPS70c showing a stronger effect, which is correlated to a certain extent with the degree of substitution.

Full Text

Effects of Sulfated Tremella Polysaccharide and Sulfated Codonopsis pilosula Polysaccharide on T Lymphocyte Proliferation and Interleukin-2 mRNA Expression in Broilers

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Abstract: To investigate the immunoenhancement mechanisms of sulfated Tremella polysaccharide (sTPS70c) and sulfated Codonopsis pilosula polysaccharide (sCPPS50c), this study examined their effects on chicken T lymphocyte proliferation and interleukin-2 (IL-2) mRNA expression using the methyl thiazolyl tetrazolium (MTT) assay and real-time fluorescent quantitative PCR, with unmodified Tremella polysaccharide (TPStp) as a control. When polysaccharides were added alone to peripheral blood lymphocytes, both sTPS70c and sCPPS50c significantly stimulated T lymphocyte proliferation at nearly all concentrations tested ($P<0.05$), whereas TPStp exhibited significant stimulation only at 3.125 μg/mL ($P<0.05$). When polysaccharides were co-administered with phytohemagglutinin P (PHA-P), sCPPS50c significantly enhanced lymphocyte proliferation at concentrations of 0.391–1.563 μg/mL ($P<0.05$). Both sTPS70c and sCPPS50c at 1.563 μg/mL significantly increased IL-2 mRNA expression in T lymphocytes ($P<0.05$), with sTPS70c demonstrating significantly stronger effects than unmodified TPStp ($P<0.05$). The most potent effect was observed for sTPS70c at 1.563 μg/mL. These findings suggest that sulfation modification enhances both lymphoproliferative activity and IL-2 mRNA expression, with sTPS70c showing superior effects that correlate to some extent with its degree of substitution.

Keywords: sulfated Tremella polysaccharide; sulfated Codonopsis pilosula polysaccharide; lymphocyte proliferation; interleukin-2

Introduction

Sulfation modification can further enhance the biological activity of polysaccharides and confer novel medicinal properties, particularly antiviral and immunomodulatory activities, making it a subject of considerable research interest. Sulfated polysaccharides exert immunoenhancement through multiple pathways and mechanisms at various levels. Previous studies have demonstrated that sulfated polysaccharides primarily modulate the immune system by activating immune cells including T lymphocytes, B lymphocytes, natural killer (NK) cells, dendritic cells, and macrophages, as well as by promoting cytokine secretion [1-3].

Interleukin-2 (IL-2) is a cytokine produced by activated T lymphocytes that promotes B lymphocyte differentiation and antibody secretion, induces interferon production, and enhances the activity of monocytes and NK cells, thereby playing a crucial regulatory role in immune responses [4-5]. Building upon our previous research [6-7], this study further investigated the immunoenhancement mechanisms of sulfated polysaccharides by examining the effects of sulfated Tremella polysaccharide (sTPS70c) and sulfated Codonopsis pilosula polysaccharide (sCPPS50c) on chicken T lymphocyte proliferation and IL-2 mRNA expression levels using MTT assay and real-time fluorescent quantitative PCR. The objective was to evaluate their immunoenhancement activity and identify the more potent sulfated polysaccharide candidate.

Materials and Methods

1.1 Reagents and Materials

RPMI-1640 medium was purchased from Gibco; fetal bovine serum from Zhejiang Tianhang Biological Technology; phytohemagglutinin P (PHA-P) from Sigma; lymphocyte separation medium from Shanghai Huajing Biological Technology; dimethyl sulfoxide (DMSO) from Tianjin Kermel Chemical Reagent; RNAiso Plus, Taq polymerase, 5× Taq buffer, and 2.5 mmol/L MgCl from TakaRa; DEPC-treated water from Nanjing Shoukang Biotechnology; SYBR Green I Master Mix from Toyobo (Japan); 10 mmol/L dNTPs from Shanghai Jiebaisi Biotechnology; IL-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers designed by Nanjing GenScript; DNA marker and spin column PCR product purification kit from Beijing Tiangen Biotech.

1.2 Polysaccharide Preparations

sTPS70c, sCPPS50c, and unmodified Tremella polysaccharide (TPStp) were provided by the Laboratory of Traditional Chinese Veterinary Medicine at Nanjing Agricultural University. Based on preliminary experiments, sTPS70c, sCPPS50c, and TPStp were diluted with serum-free RPMI-1640 medium to concentrations of 6.250, 3.125, 1.563, 0.782, and 0.391 μg/mL, sterilized through 0.22 μm microporous membranes, and stored at 4°C.

1.3 Isolation and Culture of Chicken T Lymphocytes

Peripheral blood (30 mL) was aseptically collected from the hearts of adult roosters with heparin anticoagulation, diluted with Hank' s solution, layered over lymphocyte separation medium, and centrifuged at 2,000 rpm for 20 minutes. The cloudy cell layer was harvested, washed with Hank' s solution, and centrifuged at 1,500 rpm for 10 minutes. After cell counting, the density was adjusted to 2.5×10 cells/mL. Eighty microliters of cell suspension were added to each well of 96-well plates, followed by 100 μL of sTPS70c, sCPPS50c, or

TPStp at various concentrations (four replicates per treatment). PHA-P was added to a final concentration of 20 μg/mL. Polysaccharide-only controls, cell-only controls, and PHA-P-only controls were included. Cells were incubated at 37°C with 5% CO for 48 hours, then 20 μL of MTT solution was added. After an additional 4-hour incubation, 100 μL of DMSO was added to each well and absorbance at 570 nm (A) was measured to assess T lymphocyte proliferation [7-8].

1.4 Determination of IL-2 mRNA Expression in Chicken T Lymphocytes

1.4.1 Lymphocyte Isolation and Culture Lymphocytes were isolated as described in section 1.3. The cell concentration was adjusted to 1×10 cells/mL, and 800 μL of cell suspension was added to each well of 6-well plates. One milliliter of sTPS70c, sCPPS50c, or TPStp at concentrations of 1.563–6.250 μg/mL was added, followed by 200 μL of PHA-P solution (20 μg/mL). Cell-only and PHA-P-only controls were included. After 36-hour incubation at 37°C with 5% CO , cells were collected in 1.5 mL Eppendorf tubes and centrifuged at 2,000 rpm for 10 minutes in a refrigerated centrifuge. The supernatant was discarded, cells were resuspended in 1 mL phosphate-buffered saline (PBS), centrifuged again for 10 minutes, and the pellet was stored at -70°C for RNA extraction.

1.4.2 Total RNA Extraction One milliliter of Trizol reagent was added to each Eppendorf tube, vortexed, and incubated at room temperature for 10 minutes. Two hundred microliters of chloroform were added, vigorously shaken, and left at room temperature for 10 minutes. After centrifugation at 12,000 rpm for 15 minutes at 4°C, 350 μL of the aqueous phase was transferred to a new tube, mixed with an equal volume of isopropanol, and incubated at 4°C for 20 minutes. The mixture was centrifuged at 12,000 rpm for 15 minutes at 4°C, the supernatant was discarded, and the RNA pellet was washed with 1 mL of 75% ethanol followed by centrifugation at 10,000 rpm for 5 minutes at 4°C. The pellet was air-dried and dissolved in 20 μL of 0.1% DEPC-treated water, then stored at -20°C [9-10].

1.4.3 Reverse Transcription Reverse transcription was performed using extracted RNA as template according to the kit instructions. The cDNA products were stored at -20°C.

1.4.4 Primer Design Primers for chicken IL-2 (GenBank accession: AJ224516.1) and GAPDH (GenBank accession: NM204305) were designed and synthesized by Nanjing GenScript Biotechnology to amplify 138 bp and 146 bp fragments, respectively [11].

IL-2 (138 bp):
Forward: 5' -AGGGGTGAATTCACAAGGG-3'
Reverse: 5' -ACTTCTCCCAGGTAACAC-3'

GAPDH (146 bp):
Forward: 5' -TGGAGAAACCAGCCAAGTAT-3'
Reverse: 5' -CGCATCAAAGGTGGAAGAAT-3'

1.4.5 Real-Time Fluorescent Quantitative PCR The reaction mixture contained 10 µL SYBR Green I Master Mix, 1.2 µL each of forward and reverse primers, 2 µL cDNA, and nuclease-free water to a final volume of 20 µL [12]. Thermal cycling conditions were: 95°C for 3 minutes, followed by 40 cycles of 94°C for 30 seconds and gene-specific annealing temperature for 30 seconds with fluorescence acquisition. A melting curve analysis was performed by slow heating from 60°C to 99°C.

1.5 Statistical Analysis

The relative IL-2 mRNA expression levels were calculated using the comparative Ct method (2^(-ΔΔCt)) [13]. Data are presented as mean ± standard error. Duncan' s multiple range test was performed using SPSS 16.0 to compare IL-2 mRNA expression levels among different polysaccharide treatments.

Results

2.1 Effects of sTPS70c, sCPPS50c, and TPStp on Chicken T Lymphocyte Proliferation

2.1.1 Effects of Polysaccharides Alone on T Lymphocyte Proliferation As shown in Table 1 , sCPPS50c at 0.391–6.250 µg/mL, sTPS70c at 0.782–6.250 µg/mL, and TPStp at 3.125 µg/mL exhibited significantly higher A values compared to the cell control ($P<0.05$), indicating significant stimulation of T lymphocyte proliferation. The sulfate substitution degrees of sTPS70c and sCPPS50c were 1.62 and 1.36, respectively, suggesting that their enhanced proliferative effects compared to unmodified polysaccharide are related to sulfate substitution degree.

2.1.2 Synergistic Effects of Polysaccharides with PHA-P on T Lymphocyte Proliferation Table 2 shows that sCPPS50c at 0.391–1.563 µg/mL produced significantly higher A values than the PHA-P control ($P<0.05$), demonstrating its ability to synergize with PHA-P in significantly promoting chicken T lymphocyte proliferation.

2.2 Effects on IL-2 mRNA Expression in Chicken T Lymphocytes

2.2.1 Optimization of Reaction Conditions The amplification curves exhibited typical S-shaped profiles with uniform spacing, and the standard curve showed $R^2 > 0.99$ with slope differences < 0.1, indicating consistent PCR amplification efficiency (Figure 1 [Figure 1: see original paper]). Melting curve

analysis revealed single peaks, confirming the absence of non-specific products or primer dimers (Figure 2 [Figure 2: see original paper]).

2.2.2 Effects of sTPS70c, sCPPS50c, and TPStp on IL-2 mRNA Expression Levels Using the PHA-P control as the calibrator ($2^{-\Delta\Delta Ct}$ = 1), sTPS70c at 1.563–6.250 μg/mL increased chicken T lymphocyte IL-2 mRNA expression to 1.87, 2.26, and 8.13-fold, respectively. sCPPS50c at 1.563–3.125 μg/mL increased expression to 1.80 and 3.32-fold, respectively. TPStp at 1.563 and 6.250 μg/mL increased expression to 1.52 and 1.83-fold, respectively, indicating that all polysaccharides promoted IL-2 mRNA expression.

As shown in Figure 3 [Figure 3: see original paper], sTPS70c and sCPPS50c at 1.563 μg/mL significantly elevated IL-2 mRNA expression compared to the PHA-P control ($P<0.05$). Notably, sTPS70c at 1.563 μg/mL showed significantly higher expression than TPStp ($P<0.05$). While sTPS70c at 6.250 μg/mL and both sulfated polysaccharides at 3.125 μg/mL showed higher expression than TPStp, these differences were not statistically significant ($P>0.05$). The strongest IL-2 mRNA expression was induced by sTPS70c at 1.563 μg/mL, which also had the higher sulfate substitution degree.

Discussion

Lymphocyte transformation is a direct indicator of cellular immunity [14]. Our results demonstrate that when polysaccharides were used alone, sTPS70c and sCPPS50c at nearly all concentrations significantly promoted T lymphocyte proliferation, whereas TPStp was effective only at 3.125 μg/mL. When combined with PHA-P, sCPPS50c at 1.563 μg/mL significantly enhanced proliferation. These findings indicate that sulfation modification enhances the immunological activity of polysaccharides. The stronger proliferative effect of polysaccharides alone compared to their synergistic effect with PHA-P suggests that sulfated polysaccharides can independently stimulate lymphocyte proliferation more effectively than mitogens alone. Nguyen et al. [15] reported that sulfated Auricularia auricula polysaccharides sAAP1 and sAAPt could promote peripheral blood lymphocyte proliferation either alone or in synergy with PHA-P, confirming that sulfation significantly enhances cellular immunity.

The degree of sulfate substitution is closely related to immunoenhancement activity. sTPS70c and sCPPS50c, with sulfate substitution degrees of 1.62 and 1.36, respectively, showed stronger T lymphocyte proliferative effects correlating with higher substitution degrees. Previous research demonstrated that the proliferative effect of sulfated Angelica sinensis polysaccharide on splenic lymphocytes was positively associated with its sulfate substitution degree [16], consistent with our findings. Sulfation likely introduces new structural features and alters the physicochemical properties and conformational structure of polysaccharides, thereby substantially enhancing their biological activity.

IL-2 activates multiple immune cell types, induces lymphocyte proliferation and immune effector functions, and represents a central regulator in cellular immunity [17]. Therefore, analyzing IL-2 mRNA expression levels provides a preliminary assessment of T lymphocyte activation and reflects the cellular immune status [18]. Our results show that both sulfated polysaccharides, sTPS70c and sCPPS50c, significantly promoted IL-2 mRNA expression at 1.563 μg/mL compared to the PHA-P control, though with significant differences between the two compounds. sTPS70c at 1.563 μg/mL showed significantly higher expression than TPStp, demonstrating its potent ability to promote IL-2 mRNA expression in peripheral blood lymphocytes, which would enhance IL-2 secretion and immunomodulatory function. Previous studies have shown that Astragalus polysaccharide significantly enhances IL-2, IFN- , and TNF- mRNA expression in canine splenic lymphocytes, with IL-2 and IFN- expression levels significantly surpassing those induced by concanavalin A (ConA) [19].

Both sTPS70c and sCPPS50c enhance lymphocyte proliferative activity and significantly increase IL-2 mRNA expression, with sTPS70c demonstrating superior effects. These sulfated polysaccharides represent potential immunoenhancement agents for further development.

References

[1] AHN G, BING S J, KANG S M, et al. The JNK/NFkB pathway is required to activate murine lymphocytes induced by a sulfated polysaccharide from Ecklonia cava[J]. Biochimica et Biophysica Acta (BBA): General Subjects, 2013, 1830(3): 2820–2829.

[2] YANG T H, JIA M, ZHOU S Y, et al. Antivirus and immune enhancement activities of sulfated polysaccharide from Angelica sinensis[J]. International Journal of Biological Macromolecules, 2012, 50(3): 768–772.

[3] MA X, GUO Z H, WANG D Y, et al. Effects of sulfated polysaccharides and their prescriptions on immune response of ND vaccine in chicken[J]. Carbohydrate Polymers, 2010, 82(1): 9–13.

[4] SHI L, DUAN X M, HU M L, et al. Effects of polysaccharide from Ornithogalum caudatum on T lymphocytes and IL-2 mRNA expression in mice[J]. Journal of Jilin University: Medicine Edition, 2004, 30(5): 678–680.

[5] CHEN R Q. Effects of Grifola frondosa polysaccharide on interleukin-2 and interferon- expression in human peripheral blood mononuclear cells[J]. Journal of Fujian Medical University, 2010, 44(6): 447–449.

[6] ZHAO X N, HU Y L, WANG D Y, et al. Optimization of sulfated modification conditions of tremella polysaccharide and effects of modifiers on cellular infectivity of NDV[J]. International Journal of Biological Macromolecules, 2011, 49(1): 44–49.

[7] ZHAO X N, HU Y L, WANG D Y, et al. The comparison of immune-enhancing activity of sulfated polysaccharides from Tremella and Condonpsis pilosula[J]. Carbohydrate Polymers, 2013, 98(1): 438-443.

[8] ZHANG J, CHEN J, WANG D Y, et al. Immune-enhancing activity comparison of sulfated ophiopogonpolysaccharide and sulfated jujube polysaccharide[J]. International Journal of Biological Macromolecules, 2013, 52: 212-217.

[9] WANG D Q, WANG M L, ZHAO X J, et al. Effects of glycyrrhetinic acid liposome on IL-2, IL-4 and IFN- mRNA expression in chickens in vitro[J]. Chinese Journal of Veterinary Science, 2013, 33(6): 874-877.

[10] GAO H, WANG D Y, GUO L W, et al. Effects of Epimedium polysaccharide liposome on chicken lymphocyte proliferation and IL-2, IL-4 and IFN- mRNA expression[J]. Acta Veterinaria et Zootechnica Sinica, 2013, 44(1): 115-121.

[11] LU Y, WANG K M, GUO Z H, et al. Effects of sulfated Epimedium polysaccharide on IL-2 and IFN- mRNA expression in chicken peripheral blood lymphocytes[J]. Jiangsu Journal of Agricultural Sciences, 2009, 25(5): 1073-1077.

[12] YUAN J H. Experimental study on real-time fluorescent quantitative PCR technology[J]. Modern Agricultural Science and Technology, 2010(13): 20-22.

[13] LIVAK K J, SCHMITTGEN T D. Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-ΔΔCt) method[J]. Methods, 2001, 25(4): 402-408.

[14] LI C H, SANTOSO S, LO D D, et al. Quantitative analysis of T cell homeostatic proliferation[J]. Cellular Immunology, 2007, 250(1/2): 45-54.

[15] NGUYEN T L, WANG D Y, HU Y L, et al. Immuno-enhancing activity of sulfated Auricularia auricula polysaccharides[J]. Carbohydrate Polymers, 2012, 89(4): 1117-1122.

[16] ZHAO X M, CUI B A, ZHANG H Y, et al. Effects of four traditional Chinese medicine polysaccharides on porcine splenic lymphocyte proliferation[J]. Journal of Hebei Agricultural University, 2007, 30(3): 82-85.

[17] LI Y Y, LI P G, ZHANG Y Y, et al. Progress on interleukin-2 (IL-2) application in veterinary medicine[J]. Journal of Hebei Normal University of Science & Technology, 2006, 20(4): 63-66.

[18] LUAN W M, YANG S B, GU Y B, et al. Dynamic changes of IFN- and IL-2 mRNA expression in chicken gut-associated lymphoid tissue[J]. Chinese Veterinary Science, 2011, 41(3): 292-297.

[19] QIU H H, ZHAO J, LIU F H, et al. Effects of Astragalus polysaccharide on cytokine mRNA expression in canine splenic lymphocytes[J]. Chinese Journal of Veterinary Medicine, 2006, 46(6): 6-8.

Note: Figure translations are in progress. See original paper for figures.