ChinaRxiv

AI translation · View original & related papers at
chinarxiv.org/items/chinaxiv-201812.00601

Effects of Sulfated Tremella Polysaccharide and Codonopsis pilosula Polysaccharide on Chicken T Lymphocyte Proliferation and IL-2 mRNA Expression Levels Postprint

Authors: Zhu Guangshuang, Hu Yuanliang, Cao Kan(1, Wang Benzhong

Date: 2017-11-07T00:00:00+00:00

Abstract

To investigate the immunoenhancement mechanisms of sulfated Tremella polysaccharide (sTPS70c) and sulfated Codonopsis polysaccharide (sCPPS50c), the present study employed unmodified Tremella polysaccharide (TPStp) as a control and utilized the thiazolyl blue (MTT) assay and real-time fluorescent 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 demonstrated that when polysaccharides were added alone to peripheral blood lymphocytes, sTPS70c and sCPPS50c at nearly all concentrations significantly stimulated 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 ranging from 0.391 to 1.563 g/mL ($P<0.05$). Both sTPS70c and sCPPS50c at a concentration of 1.563 g/mL enhanced IL-2 mRNA expression in T lymphocytes ($P<0.05$), with sTPS70c exhibiting a significantly stronger promoting effect on IL-2 mRNA expression compared to unmodified TPStp ($P<0.05$), and this effect was most potent at the concentration of 1.563 g/mL. These findings suggest that sulfated modification can enhance the lymphocyte proliferation activity of polysaccharides and markedly increase IL-2 mRNA expression, with sTPS70c demonstrating stronger effects, which correlates to a certain extent with the degree of substitution.

ChinaRxiv 中X

Full Text

Effect of Sulfated Polysaccharides from *Tremella* and *Codonopsis pilosula* on T Lymphocyte Proliferation and Interleukin-2 mRNA Expression in Chickens

ZHU Guangshuang[1], HU Yuanliang[2]*, CAO Kan[1], WANG Benzhong[1]
(1. College of Biological Engineering, Wuhu Institute of Technology, Wuhu 241003, China;
2. College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China)

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 added alone to peripheral blood lymphocytes, sTPS70c and sCPPS50c significantly stimulated T lymphocyte proliferation at nearly all concentrations tested ($P<0.05$), whereas TPStp only showed significant stimulation at 3.125 μg/mL ($P<0.05$). When administered concurrently 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 significantly upregulated IL-2 mRNA expression in T lymphocytes at 1.563 μg/mL ($P<0.05$), with sTPS70c demonstrating significantly stronger effects than unmodified TPStp ($P<0.05$). The effect of sTPS70c was most pronounced at 1.563 μg/mL. These findings suggest that sulfation modification enhances lymphoproliferative activity and markedly increases IL-2 mRNA expression, with sTPS70c exhibiting superior activity that correlates to some extent with its degree of sulfate 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 by endowing or strengthening antiviral and immunomodulatory activities, which has attracted considerable research attention. Sulfated polysaccharides exert immunoenhancing effects through multiple pathways and mechanisms. Studies have demonstrated that sulfated polysaccharides primarily regulate the immune system by activating immune cells such as 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, playing a crucial regulatory role in immune responses[4-5]. Therefore, based on previous studies[6-7], this experiment employed the methyl thiazolyl tetrazolium (MTT) assay and real-time fluorescent quantitative PCR to investigate the effects of sulfated *Tremella* polysaccharide (sTPS70c) and sulfated *Codonopsis pilosula* polysaccharide (sCPPS50c) on chicken T lymphocyte proliferation and IL-2 mRNA expression, aiming to evaluate their immunoenhancing activities and identify the more potent sulfated polysaccharide.

1. Materials and Methods

1.1 Reagents RPMI1640 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 Development; dimethyl sulfoxide (DMSO) from Tianjin Kemiao Chemical Reagent; RNAiso Plus, Taq polymerase, 5×Taq buffer, and 2.5 mmol/L magnesium chloride (MgCl) from TakaRa; DEPC-treated water from Nanjing Shoukang Biological Technology; SYBR Green I Master Mix from Toyobo (Japan); 10 mmol/L deoxynucleotide triphosphates (dNTPs) from Shanghai Jiebeisi 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 Experimental Polysaccharides 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 RPMI1640 medium to five concentrations: 6.250, 3.125, 1.563, 0.782, and 0.391 µg/mL, sterilized by filtration through a 0.22 µm microporous membrane, 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 using heparin as an anticoagulant, diluted with Hank' s solution, and layered over lymphocyte separation medium. After centrifugation at 2,000 r/min for 20 minutes, the cloudy intermediate cell layer was harvested, washed with Hank' s solution, and centrifuged at 1,500 r/min for 10 minutes. Following cell counting, the density was adjusted to 2.5×10 cells/mL. Subsequently, 80 µL of cell suspension was added to each well of a 96-well culture plate, followed by 100 µL of sTPS70c, sCPPS50c, or TPStp at various concentrations, with four replicate wells per treatment. Phytohemagglutinin P (PHA-P) was added to a final concentration of 20 µg/mL where indicated. Polysaccharide controls, cell controls, and PHA-P controls were also included. The plates were incubated at 37 °C with 5% CO for 48 hours, after which 20 µL of MTT solution was added. Following an additional 4-hour incubation, 100 µL of DMSO was added to each well, and absorbance at 570 nm (A) was measured using an enzyme-linked immunosorbent assay reader 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 The isolation procedure was identical to that 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 a 6-well culture plate. Subsequently, 1 mL of sTPS70c, sCPPS50c, or TPStp at concentrations ranging from 1.563 to 6.250 µg/mL was added, followed by 200 µL of PHA-P solution (20 µg/mL). Cell controls and PHA-P controls were also prepared. After incubation at 37 °C with 5% CO for 36 hours, cells were harvested into 1.5 mL Eppendorf tubes and centrifuged at 2,000 r/min for 10 minutes in a refrigerated centrifuge. The supernatant was discarded, and the pellet was resuspended in 1 mL of phosphate-buffered saline (PBS), followed by another 10-minute centrifugation. After removing the supernatant, the cell pellets were stored at -70 °C for subsequent RNA extraction.

1.4.2 Total RNA Extraction One milliliter of Trizol reagent was added to each Eppendorf tube, vortexed thoroughly, and incubated at room temperature for 10 minutes. Subsequently, 200 µL of chloroform was added to each tube, vigorously shaken, and left at room temperature for 10 minutes. After centrifugation at 12,000 r/min for 15 minutes at low temperature, 350 µL of the upper 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 then centrifuged at 12,000 r/min for 15 minutes at low temperature, the supernatant was discarded, and the RNA pellet was washed with 1 mL of 75% ethanol. Following a 5-minute centrifugation at 10,000 r/min at 4 °C, the supernatant was removed, and the RNA pellet was air-dried at room temperature. The RNA was dissolved in 20 µL of 0.1% DEPC-treated water and stored at -20 °C[9-10].

1.4.3 Reverse Transcription Reverse transcription was performed using the extracted RNA as template according to the manufacturer' s instructions for the reverse transcription kit. The resulting cDNA products were stored at -20 °C for subsequent use.

1.4.4 Primer Design Based on the chicken IL-2 (GenBank accession: AJ224516.1) and GAPDH (GenBank accession: NM204305) RNA sequences, primers were synthesized by Nanjing GenScript Biotechnology to amplify 138 bp and 146 bp fragments of IL-2 and GAPDH, respectively[11].

IL-2 (138 bp): Forward primer, 5' -AGGGGTGAATTCACAAGGG-3' ; Reverse primer, 5' -ACTTCTCCCAGGTAACAC-3' .

GAPDH (146 bp): Forward primer, 5' -TGGAGAAACCAGCCAAGTAT-3' ; Reverse primer, 5' -CGCATCAAAGGTGGAAGAAT-3' .

1.4.5 Real-Time Fluorescent Quantitative PCR The reaction mixture contained 10 µL of SYBR Green I Master Mix, 1.2 µL of forward and reverse primers, and 2 µL of cDNA, brought to a final volume of 20 µL with triple-distilled water[12].

The thermal cycling conditions were as follows: initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 94 °C for 30 seconds and annealing at the specific temperature for 30 seconds with fluorescence signal acquisition. A melting curve analysis was performed by gradually increasing the temperature from 60 °C to 99 °C.

1.5 Statistical Analysis The relative IL-2 mRNA expression levels in each sample were calculated using the comparative Ct method[13]. Data are presented as mean ± standard error and were analyzed using Duncan' s multiple range test in SPSS 16.0 to compare IL-2 mRNA expression levels in T lymphocytes treated with different polysaccharides.

2. Results and Analysis

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

2.1.1 Effects of Polysaccharides Alone As shown in Table 1 , the A values for sCPPS50c at concentrations of 0.391-6.250 µg/mL, sTPS70c at 0.782-6.250 µg/mL, and TPStp at 3.125 µg/mL were significantly higher than those of the cell control ($P<0.05$). The sulfate substitution degrees of sTPS70c and sCPPS50c were 1.62 and 1.36, respectively, indicating that their ability to promote chicken T lymphocyte proliferation at the indicated concentrations was stronger than that of the unmodified polysaccharide and correlated with sulfate substitution degree.

Table 1 Effects of polysaccharides alone on proliferation of chicken T lymphocytes

In the same row, values with different letter superscripts indicate significant differences (P<0.05). The same applies to the following tables.

2.1.2 Effects of Polysaccharides in Combination with PHA-P As shown in Table 2 , the A values for sCPPS50c at concentrations of 0.391-1.563 µg/mL were significantly higher than those of the PHA-P control ($P<0.05$), indicating that sCPPS50c significantly promoted chicken T lymphocyte proliferation in synergy with PHA-P at these concentrations.

Table 2 Effects of polysaccharides in combination with PHA-P on proliferation of chicken T lymphocytes

2.2 Effects on IL-2 mRNA Expression Levels

2.2.1 Optimization of Reaction Conditions The amplification curves exhibited a characteristic S-shape with uniform spacing between curves, and the standard curve showed $R^2>0.99$ with a slope difference of <0.1, indicating consistent PCR amplification efficiency (Figure 1 [Figure 1: see original paper]). Melting curve analysis revealed single peaks, confirming the absence of nonspecific products or primer dimers (Figure 2 [Figure 2: see original paper]).

Figure 1 Sample amplification curve and standard curve

Figure 2 Sample melting curve

2.2.2 Effects of sTPS70c, sCPPS50c, and TPStp on IL-2 mRNA Expression With the PHA-P control set as 1 ($2^{\wedge}(-\Delta\Delta Ct)$), sTPS70c at concentrations of 1.563–6.250 µg/mL increased chicken T lymphocyte IL-2 mRNA expression levels by 1.87-, 2.26-, and 8.13-fold; sCPPS50c at 1.563–3.125 µg/mL increased expression by 1.80- and 3.32-fold; and TPStp at 1.563 and 6.250 µg/mL increased expression by 1.52- and 1.83-fold, respectively, demonstrating 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 increased chicken T lymphocyte IL-2 mRNA expression compared with the PHA-P control ($P<0.05$). sTPS70c at 1.563 µg/mL showed significantly higher expression than TPStp ($P<0.05$). Although sTPS70c at 6.250 µg/mL and both sTPS70c and sCPPS50c at 3.125 µg/mL exhibited higher expression than TPStp, the differences were not significant ($P>0.05$). sTPS70c demonstrated the strongest IL-2 mRNA expression at 1.563 µg/mL, which correlated with its higher sulfate substitution degree.

Figure 3 IL-2 mRNA expression levels

Discussion

Lymphocyte transformation is the most direct indicator of cellular immunity[14]. The present results demonstrate that when stimulating T lymphocytes alone, sTPS70c and sCPPS50c significantly promoted proliferation at nearly all concentrations, whereas TPStp only showed significant effects at 3.125 µg/mL. When combined with PHA-P, sCPPS50c significantly enhanced T lymphocyte proliferation at 1.563 µg/mL. These findings indicate that sulfation modification enhances the immunological activity of polysaccharides. The proliferative effect on lymphocytes without PHA-P stimulation was stronger than that observed with polysaccharide-PHA-P co-treatment, suggesting that sulfated polysaccharides can independently stimulate lymphocyte proliferation with greater efficacy than mitogens. Nguyen et al.[15] reported that sulfated *Auricularia auricula* polysaccharides sAAP1 and sAAPt could promote peripheral blood lymphocyte proliferation either alone or in combination with PHA-P, demonstrating that sulfation significantly enhances cellular immune function.

The degree of sulfate substitution is closely related to the immunoenhancing activity of polysaccharides. sTPS70c and sCPPS50c had sulfate substitution degrees of 1.62 and 1.36, respectively, and their ability to promote T lymphocyte proliferation was associated with higher sulfate substitution. Previous studies have shown that the proliferative effect of sulfated *Angelica sinensis* polysaccharide on splenic lymphocytes correlates with its degree of sulfate substitution, with higher substitution conferring stronger effects[16], consistent with our findings. Sulfation may generate novel polysaccharide structures, altering their physicochemical properties and stereoconformation, thereby substantially enhancing biological activity.

IL-2 activates multiple immune cell types, induces lymphocyte proliferation and immune effector functions, and represents a central mediator of immunoregulation in cellular immunity[17]. Therefore, analyzing IL-2 mRNA expression levels enables preliminary evaluation of T lymphocyte activation in peripheral blood and reflects the organism' s cellular immune status[18]. Our results show that both sulfated polysaccharides, sTPS70c and sCPPS50c, significantly increased IL-2 mRNA expression compared with the PHA-P control at 1.563 µg/mL, promoting IL-2 mRNA expression in T lymphocytes. However, the two sulfated polysaccharides differed significantly in their expression levels. sTPS70c at 1.563 µg/mL showed significantly higher IL-2 mRNA expression than TPStp, demonstrating robust promotion of IL-2 mRNA expression in peripheral blood lymphocytes. This abundant expression would enhance IL-2 secretion and exert immunostimulatory effects. Studies have shown that Astragalus polysaccharide significantly enhances IL-2, interferon- (IFN-), and tumor necrosis factor- (TNF-) mRNA expression in canine splenic lymphocytes, with IL-2 and IFN- mRNA expression levels significantly surpassing those induced by concanavalin A (ConA)[19].

Both sTPS70c and sCPPS50c enhance lymphoproliferative activity and significantly increase IL-2 mRNA expression, with sTPS70c demonstrating superior effects, suggesting their potential as immunoenhancing 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] 石磊, 段秀梅, 胡梦林, 等. 虎眼万年青多糖对小鼠 T 细胞及其细胞因子 IL-2 mRNA 表达的影响 [J]. 吉林大学学报: 医学版, 2004, 30(5): 678-680.

[5] 陈瑞庆. 灰树花多糖对人外周血单个核细胞白细胞介素-2 及干扰素- 表达的影响 [J]. 福建医科大学学报, 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 *Codonopsis 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] 王德清, 王梦琳, 赵晓娟, 等. 甘草次酸脂质体体外对鸡 IL-2、IL-4 和 IFN- mRNA 表达的影响 [J]. 中国兽医学报, 2013, 33(6): 874-877.

[10] 高焕, 王德云, 郭利伟, 等. 淫羊藿多糖脂质体对鸡淋巴细胞增殖及 IL-2、IL-4 和 IFN- mRNA 表达的影响 [J]. 畜牧兽医学报, 2013, 44(1): 115-121.

[11] 卢宇, 王凯民, 郭振环, 等. 硫酸化淫羊藿多糖对鸡外周血淋巴细胞 IL-2 和 IFN- 的 mRNA 表达的影响 [J]. 江苏农业学报, 2009, 25(5): 1073-1077.

[12] 袁继红. 实时荧光定量 PCR 技术的实验研究 [J]. 现代农业科技, 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] 赵现敏, 崔保安, 张红英, 等. 4 种中药多糖对猪脾淋巴细胞增殖的影响 [J]. 河北农业大学学报, 2007, 30(3): 82-85.

[17] 李蕴玉, 李佩国, 张艳英, 等. 白细胞介素-2(IL-2) 在兽医中的应用进展 (综述)[J]. 河北科技师范学院学报, 2006, 20(4): 63-66.

[18] 栾伟民, 杨树宝, 顾玉宝, 等. 鸡肠相关性淋巴组织中 IFN- 和 IL-2 mRNA 表达的动态变化 [J]. 中国兽医科学, 2011, 41(3): 292-297.

[19] 邱河辉, 赵娟, 刘凤华, 等. 黄芪多糖对犬脾淋巴细胞细胞因子 mRNA 表达的影响 [J]. 中国兽医杂志, 2006, 46(6): 6-8.

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

Source: ChinaXiv —Machine translation. Verify with original.