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Technical Note

The biological activity of a recombinantly expressed (His)₆-tagged peanut allergen (rAra h 1) is unaffected by endotoxin removalLouise Bjerremann Jensen^{a,b,*}, Anna Maria Torp^a, Sven Bode Andersen^a, Per Stahl Skov^b, Lars K. Poulsen^b, Edward F. Knol^c, Els van Hoffen^{c,*}^a Department of Agricultural Sciences, Faculty of Life Sciences, University of Copenhagen, Denmark^b Laboratory of Medical Allergology, Allergy Clinic, National University Hospital, Copenhagen, Denmark^c Department of Dermatology and Allergology, University Medical Center, Utrecht, The Netherlands

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Abbreviations:

HR Histamine release

IL Interleukin

LPS Lipopolysaccharide

TLR Toll-like receptor

TNF Tumor necrosis factor

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ABSTRACT

The application of recombinant (His)₆-tagged proteins in cell culture assays is associated with problems due to lipopolysaccharide (LPS) contamination. LPS stimulates cells of the immune system, thereby masking antigen-specific activation of T cells. Due to the affinity of LPS for histidine it is associated with difficulties to remove LPS from recombinant (His)₆-tagged proteins. Here we describe that the Triton X-114 phase separation method can be used to remove LPS from (His)₆-tagged proteins and that the recombinant proteins retain their biological activity.

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1. Introduction

Gram-negative bacteria such as *Escherichia coli* (*E. coli*) are often used as expression organisms in the production of recombinant proteins. LPS (also known as endotoxin) from the bacterial cell wall is present in protein preparations purified from the bacteria and may influence results in cell culture assays where the recombinant proteins are used as antigens to measure antigen-specific stimulation of T cells.

LPS affects both the cytokine production and proliferation pattern in T cell and potentially other biological assays. The production of the pro-inflammatory cytokine, TNF- α , from monocytes is induced by LPS-mediated stimulation of Toll-like receptors on these cells (Simon et al., 2007). LPS also enhances the IL-10 production from monocytes, which is further enhanced by TNF- α (Daftarian et al., 1996).

The effect of LPS on proliferation is dose-dependent. Low concentrations (in pg/mL) enhance the antigen-specific cell proliferation (Cirkovic et al., 2007). However, higher concentrations (in μ g/mL) suppress this proliferation (Chen et al., 2002), which can occur via monocyte-derived IL-10 (Mielcarek et al., 1998).

The application of recombinant antigens contaminated with LPS from the expression organism may therefore cause non-specific cytokine production and affect T cell proliferation, thereby masking the antigen-specific cell response. Moreover, in murine systems LPS will stimulate B-cells independently from the other cell types, and its presence may cause a strong bias in the B-cell response of lymphocyte cultures (Rodo et al., 2006). It is therefore crucial to remove LPS from recombinant proteins before analyzing antigen-specific responses (Cirkovic et al., 2007).

Many recombinant proteins are constructed with a (His)₆-tag for easy purification from bacteria. This complicates the removal of LPS from the proteins since LPS has affinity for histidine (Matsumae et al., 1990). We show that the Triton X-114 phase separation method (Liu et al., 1997) can be used to remove LPS from (His)₆-tagged recombinant proteins. The method was tested on five recombinant protein preparations and a reduction of LPS to <0.25 EU/mL was achieved without considerable loss of protein. Here the results obtained with the recombinant Ara h 1 protein (rAra h 1, peanut allergen) are described.

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2. Materials and methods

2.1. Production of recombinant (His)₆-tagged Ara h 1 (rAra h 1)

RNA was isolated from peanut seeds (*Arachis hypogaea*, variety Gregory, NC Crop Improvement Association, NC, USA) and subsequently RT-PCR with Ara h 1 specific primers (Ara h 1 F (NheI) 5'-GTAGATGCTAGCCGTCACCTCCAGGGGAGCGG-3', Ara h 1 R (NotI) 5'-GCGATAGCGGCCGCTTAAAGCCTTCAAAATTGA-3') was performed. The RT-PCR products were cloned into pET-21b(+) (Novagen, Merck Denmark, Glostrup, Denmark) expression vectors via T/A cloning into pTZ57R/T vector (Fermentas, Helsingborg, Sweden) and blue/white colony screening. The pET-21b(+) plasmids were transfected into chemically-competent BL21-CodonPlus®(DE3)-RIL *E. coli* cells for protein expression. Protein expression was initiated by IPTG and cells were harvested after 4 h culture. Bacteria were lysed and sonicated and rAra h 1 was purified on Ni-NTA columns (Qiagen Nordic, Ballerup, Denmark) followed by buffer exchange and salt depletion on PD-10 Desalting columns (GE Healthcare, Hillerød, Denmark).

2.2. Depletion of LPS

LPS from rAra h 1 was removed by Triton X-114 phase separation as described by Liu et al. (1997). Triton X-114 was added to the proteins to a final concentration of 1% and incubated for 30 min at 4 °C with constant stirring, followed by 10 min incubation at 37 °C and a centrifugation step at 16,000 ×g and 25 °C, for 10 min. Six cycles of Triton X-114 phase separation was performed for a sufficient LPS depletion. Trace amounts of Triton X-114 were removed by dialysis against phosphate-buffered saline. Subsequently, LPS depletion was confirmed by limulus amoebocyte lysate (LAL) assay (Cambrex Bio Science, Walkersville, MD).

2.3. LPS effect on IL-10 and TNF-α production

Stimulation for 24 h of peripheral blood mononuclear cells (PBMCs) with LPS results in an increased production of TNF-α and IL-10 (Simon et al., 2007) and this can be used as an easy assay for LPS content in protein samples. PBMCs were purified from venous blood from four healthy donors by Ficoll gradient centrifugation and cultured (37 °C and 5% CO₂) in 96-well round-bottom plates in triplicate (2 × 10⁵ cells/well) in culture medium (IMDM medium containing 5% human serum (HS), penicillin (100 IU/L), streptomycin (100 mg/mL), and glutamine (1 mmol/L)) in the presence and absence of LPS-contaminated rAra h 1, rAra h 1 after three and six Triton

X-114 cycles, and LPS-free crude peanut extract (CPE) as control. After 24 h of culture, supernatants were sampled for IL-10 and TNF-α determination.

2.4. CPE-specific T cell lines (TCLs)

PBMCs were cultured in 48-well flat-bottom plates in triplicate (10⁶ cells/well) in culture medium in the presence of CPE (50 µg/mL). The proliferation of CPE-specific T cells was promoted by IL-2 addition (10 U/mL) at day 7. At day 11, TCLs were restimulated non-specifically in a 24-well flat-bottom plate with feeder mix containing allogenic PBMCs of 2 different donors (5 × 10⁵ cells/well each), and EBV-transformed B-cells (10⁵ cells/well), IL-2 (10 IU/mL), and PHA as mitogen (0.5 µg/mL). The feeder mix was irradiated before use. At day 21, antigen-specificity of TCLs was tested by stimulation in 96-well round-bottom plates (3 × 10⁴ cells/well) together with irradiated autologous PBMCs (1 × 10⁵ cells/well) either in the absence or presence of proteins (CPE, LPS-contaminated rAra h 1, and rAra h 1 after six Triton X-114 cycles, all at 25 µg/mL). Supernatants were sampled after 48 h for cytokine determinations and 0.75 µCi/well of [³H]-TdR was added. Eight hours later the cells were harvested and proliferation was determined as described previously (Ruiter et al., 2006).

Proliferation is expressed as stimulation index (SI, defined as the ratio of antigen-specific proliferation over background proliferation). A SI >2 is considered positive.

2.5. Measurement of cytokine production

The production of IL-10, TNF-α, IL-13 and IFN-γ was measured by ELISA using PeliPair™ reagent set (Sanquin, Amsterdam, The Netherlands) according to the manufacturer's instructions.

2.6. Histamine release (HR)

The biological activity of the recombinant peanut allergen after LPS depletion was tested in HR. Stripped PBMCs purified from two blood bank buffy coats were passively sensitized with serum from a peanut allergic patient or a healthy control. The cells were stimulated with rAra h 1 before and after LPS depletion. The released histamine was measured spectrofluorometrically at RefLab ApS, Denmark.

3. Results

The sequence of the cloned peanut allergen was verified by sequencing (MWG Biotech, Germany) and the expression of rAra h 1

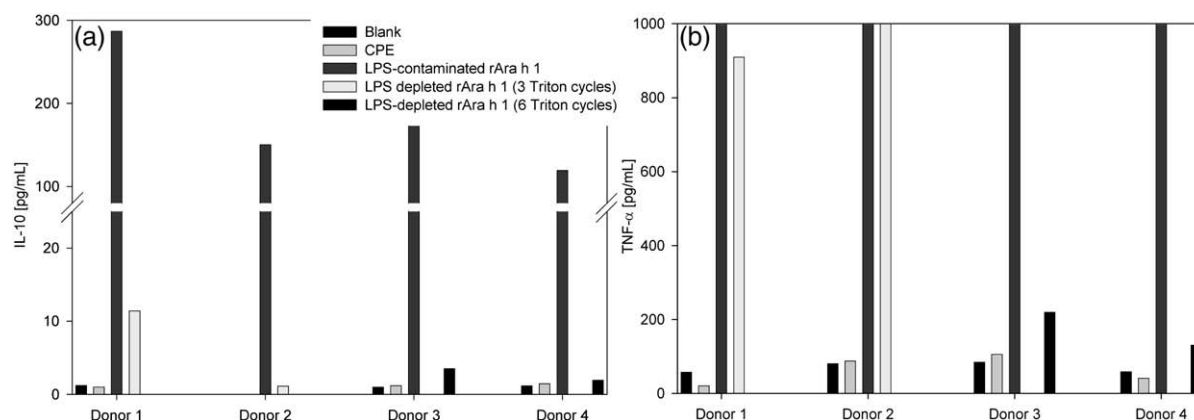


Fig. 1. The production of IL-10 (a) and TNF-α (b) by PBMCs from four healthy donors after 24 h of culture. PBMCs were stimulated with LPS-contaminated rAra h 1, before and after 3 cycles (donors 1 and 2) and 6 cycles (donors 3 and 4) of Triton X-114 phase separation, respectively. CPE was included as a LPS-free control. The TNF-α concentrations after stimulation with LPS-contaminated rAra h 1 were all above the cut-off level (1000 pg/mL) of the ELISA.

by the bacteria and subsequent purification was confirmed by SDS-PAGE (data not shown).

The amount of LPS in the rAra h 1 samples was reduced after 6 cycles of Triton X-114 phase separation from >10 EU/mL to <0.25 EU/mL, which is the FDA endotoxin limit for drugs (data not shown).

Cytokine production after 24 h stimulation of PBMCs is depicted in Fig. 1. After three Triton X-100 cycles, IL-10 production was already reduced to background levels (donors 1 and 2). The level of TNF- α is more sensitive to polluting LPS in the protein preparations. Six Triton X-114 cycles were necessary to reduce the induction of TNF- α sufficiently (donors 3 and 4).

The effect of LPS on CPE-specific TCLs from two healthy donors (A and B) is shown in Fig. 2. The TCLs of both donors did proliferate upon CPE stimulation, but not upon stimulation with LPS-contaminated

rAra h 1 (Fig. 2a, A-1 and B-1). However, they both showed IL-10 production (Fig. 2b, A-1 and B-1) and high levels of TNF- α production upon stimulation with the LPS-contaminated rAra h 1 (Fig. 2c, A-1 and B-1).

After LPS depletion, rAra h 1 no longer induced IL-10 production (Fig. 2b, A-2 and B-2). The production of TNF- α , IFN- γ and IL-13 now correlated with proliferation; the TCL of donor A proliferated to CPE and to rAra h 1, and produced TNF- α , IFN- γ and IL-13 both upon CPE and rAra h 1 stimulation, as compared to blank. The TCL of donor B proliferated to CPE but not to rAra h 1, and produced these cytokines upon CPE but not rAra h 1 stimulation (Fig. 2c, d, e, A-2 and B-2).

No difference in the ability to induce IgE-mediated histamine release in passively sensitized basophils was observed between LPS-

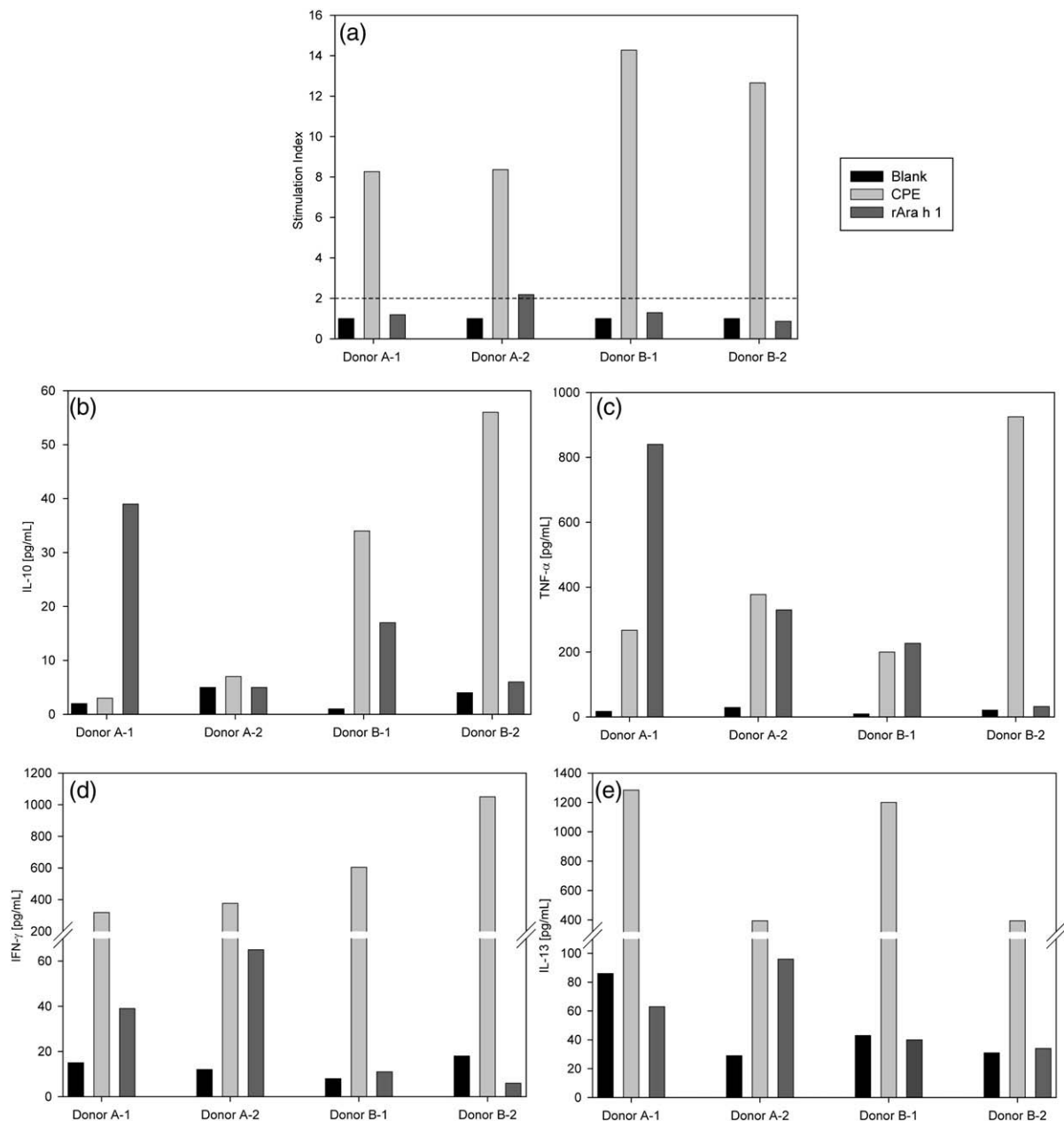


Fig. 2. Proliferation (a), and the production of IL-10 (b), TNF- α (c), IFN- γ (d), and IL-13 (e) are shown from CPE-specific TCLs of two healthy donors. TCLs were stimulated with CPE, LPS-contaminated rAra h 1 (donors A-1 and B-1) and LPS-depleted rAra h 1 (after six Triton cycles) (donors A-2 and B-2). Proliferation is expressed as SI, and is considered positive when SI > 2 (dotted line).

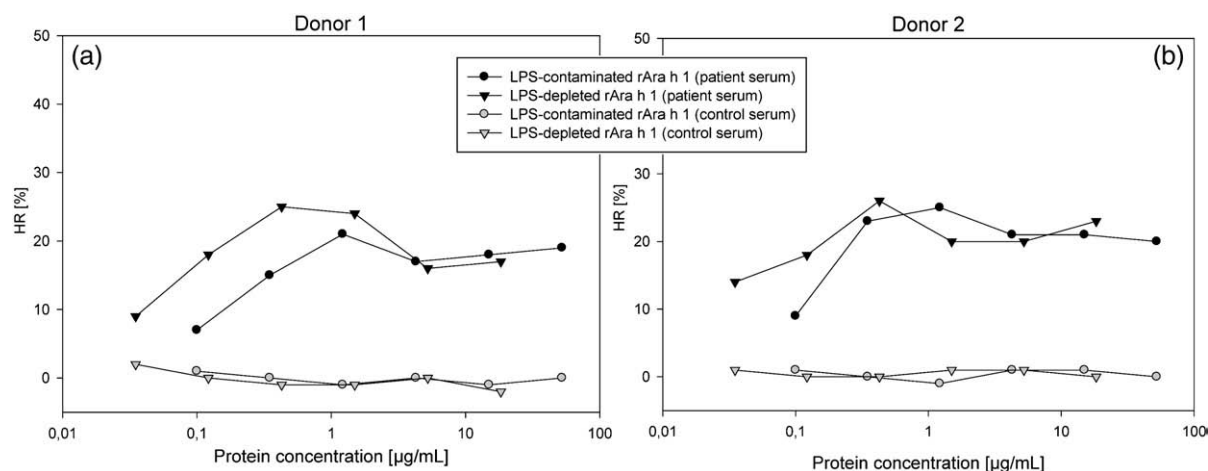


Fig. 3. The relative amount of IgE-mediated HR by passively sensitized basophils. Basophils of two different buffy coat donors (a and b) were sensitized with either serum from a peanut allergic patient or with serum from a healthy control, and stimulated with LPS-contaminated rAra h 1 or rAra h 1 depleted of LPS (after 6 cycles of Triton X-114 phase separation). HR is expressed as percentage of the total amount of histamine in the cells, which is measured after cell lysis with perchloric acid.

contaminated rAra h 1 and rAra h 1 after six Triton X-114 cycles on either of the buffy coat donors (Fig. 3).

4. Discussion

In 1997, a method was described to remove LPS from recombinant proteins (Liu et al., 1997). Based on this method we now show that it is also possible to remove LPS from (His)₆-tagged recombinant proteins. Six cycles of Triton X-114 phase separation was needed to achieve a complete removal of LPS, compared to Liu et al. where 3 cycles was sufficient. The need for more cycles in our system might be explained by the affinity of LPS for histidine (Matsumae et al., 1990). Due to this affinity, a stronger interaction of LPS and the (His)₆-tagged recombinant proteins must be overcome.

The capability of rAra h 1 to induce IgE-mediated basophil histamine release was retained upon removal of LPS. Our data in antigen-specific TCLs showed that upon stimulation with CPE, which is LPS-free, proliferation and cytokine production correlate with each other. In contrast, T cells stimulated with LPS-contaminated rAra h 1 did not proliferate and had an altered cytokine profile with high TNF- α and IL-10 production, suggestive of monocyte activation. The suppression of T cell reactivity may be explained by the monocyte-derived IL-10 (Mielcarek et al., 1998). The correlation between proliferation and cytokine responses towards rAra h 1 stimulation was restored upon LPS depletion. Together with the histamine release data, this confirmed that the depletion of LPS did not change the biological activity of the recombinant peanut allergen.

Based on our results we can conclude that we succeeded in removing LPS from (His)₆-tagged proteins, enabling the application of these recombinant proteins in cell culture assays for the measurement of antigen-specific activation of T cells and that these proteins retained

their biological/allergic activity as demonstrated by the induction of histamine release.

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