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The immunostimulatory effect of the recombinant apalbumin 1–major honeybee royal jelly protein–on TNFα release

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Abstract

Apalbumin1 (Apa1) is the major royal jelly (RJ) and honey glycoprotein having various biological properties. We have previously demonstrated that Apa1 is a regular component of honey and honeybee pollen and stimulates macrophages to release tumor necrosis factor α (TNFα). The recombinant Apa1 (rApa1) and its four recombinant protein fragments derived on the basis of partial tryptic products of Apa1 were prepared by heterologous expression in *Escherichia coli* BL21-CodonPlus(DE3)-RIL. L-Arginine at 50 mM concentration was used for improving the recombinant protein solubility. We report that the proteinous moiety of glycoprotein is responsible for stimulation of TNFα production by murine peritoneal macrophages. Moreover, we have shown that immunostimulatory effect is significantly increased after partial tryptic digestion of Apa1. It has been determined that recombinant N-terminal fragment of Apa1 is the most active elicitor of TNFα release in comparison to other three protein fragments of Apa1, as well as to the native Apa1 and rApa1.

Furthermore, it was found that native honey was able to stimulate TNFα secretion from murine macrophages, whereas the deproteinized honey had no effect on the release of TNFα. This result suggests that immunostimulatory effect of honey is based on its RJ-protein content, primarily on its dominant protein Apa1.

Keywords: Honeybee protein; Recombinant protein; Royal jelly; Honey; TNFα; Macrophages

1. Introduction

Royal jelly (RJ) is a part of the diet of honeybee larvae and determines the molecular events of cell differentiation of larvae into queens or workers [1]. There is growing scientific evidence to support the concept that the most promising bioactive compounds found in honeybee products are proteins of RJ [2–4]. The broad spectrum of physiological activity of the honeybee proteins may be demonstrated on proteins of larval diet, particularly on proteins of RJ. Systematic research of RJ-proteins at molecular level has shown that the main part of the RJ-protein fraction belongs to one protein family [5], wherein the most abundant RJ-protein apalbumin1 (Apa1) occupies an exclusive position because it is simultaneously synthesized in honeybee brain [6] as well as in hypopharyngeal glands of adult honeybee [7,8].

Abbreviations: RJ, royal jelly; Apa1, apalbumin1; rApa1-F, recombinant apalbumin1-fragment.

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For monitoring immunomodulating activity of the novel potential immunomodulators, macrophages and monocytes represent a suitable model [9]. Macrophages, which can be activated by various stimuli such as endotoxin, bacteria, viruses, or certain chemicals, play a pivotal role in the initiation and maintenance of the immune response through the production of inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)-1 and IL-6, chemokines (IL-8), as well as reactive oxygen and nitrogen intermediates [10,11]. It is supposed that Apa1 plays an important role in queen and larval honeybee nutrition and development [12]. It was shown that RJ and honey possessed potential immunomodulatory effects [2,13–15]. Apa1 is a dominant glycoprotein of RJ with a mass of 55 kDa [5,7] that has been found to be a regular component of honey [4].

However, its function in honeybee colony appears to be other than nutritional [4]. Moreover, it has been reported that some biological effects can be attributed to Apa1 [16–18]. It enhances proliferation of hepatocytes [16]. Peptides derived from Apa1 as a result of its enzymatic hydrolysis in gastrointestinal tract possess potent antihypertensive activity [17]. Furthermore, C-terminal of Apa1 may be also a precursor form of the antimicrobial peptides jelleines [18].

Recently, we have found that RJ-proteins, mainly Apa1 and Apa2, stimulate murine macrophages to release TNFα [4]. An interesting observation has been made that the stimulatory effect of the oligomeric form of Apa1 with molecular weight (MW) ca. 420 kDa was rather low in comparison to a pronounced effect of its monomeric form (MW ca. 55 kDa). A possible explanation is that amino acid sequential motifs of the Apa1 molecule, which are responsible for stimulation of TNFα release, are blocked in its oligomeric form by protein–protein interactions.

The purpose of the present study was to investigate which structural part of Apa1, proteinous or saccharidic, predominantly affects its ability to influence production of TNFα. For this purpose, recombinant Apa1 (rApa1) was prepared by heterologous expression in *Escherichia coli* and its immunostimulatory effect was compared to that of the native glycoprotein. Further, contribution of distinct moieties of Apa1 molecule on the release of TNFα was investigated employing recombinant protein fragments (rApa1-F) derived from the molecule of Apa1. In addition, we have confirmed the Apa1 present in honey is responsible for activation of murine macrophages and production of TNFα.

2. Materials and methods

2.1. Partial trypsin digestion of native Apa1

Partial trypsinolysis was performed as follows: lyophilized native Apa1 (1 mg) prepared by the method of Šimúth [19] was dissolved in 1 ml incubating buffer solution (0.02 M Tris–HCl, 0.05 M NaCl, pH 8.0) and incubated in a water bath at 37 °C with 0.001% (w/w) trypsin (Serva, Heidelberg, Germany) for different times 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 5 h. At the end of each incubation intervals, the digested samples were heated for 15 min in a boiling-water bath, examined by SDS-PAGE, and analyzed by Western blot. In the products obtained upon 3.5 h of digestion, N-terminal amino acid sequences were determined.

2.2. N-terminal amino acid sequencing

Proteins recovered upon 3.5 h tryptic digestion were separated by SDS-PAGE and electroblotted onto PVDF membranes ProBlott (Applied Biosystems, Foster city, CA, USA). The transfer was performed in an electroblotting buffer solution consisting of 10 mM (3-[cyclohexylamino]-1-propanesulfonic acid) in 10% methanol according to the procedure recommended by the manufacturer. Staining was performed using Coomassie Blue R 250. The band of interest was excised and subjected to sequencing by automated Edman degradation using an LF3600D Protein Sequencer (Beckman, USA).

2.3. Construction and expression of recombinant plasmids

The clone pRJP120 containing the complete cDNA encoding Apa1 was identified by immunoscreening of cDNA library of the nurse honeybee heads in lambda ZAP vector [20] employing antibodies against the water-soluble RJ-proteins. Recombinant plasmid pBluescript SK containing Apa1 cDNA was derived from the phage clone an in vivo excision procedure [5].

For construction of the recombinant plasmids, polymerase chain reaction (PCR) procedure was applied. The forward primers contain *NcoI* restriction endonuclease sites (underlined) including start codon. The reverse primers include *XhoI* restriction endonuclease sites (underlined) (Table 1). PCR experiments were performed with *Pfu* polymerase at different annealing temperatures: 55 °C (for DNA fragment 1, 3, and 4) or 49 °C (for DNA fragment 2). The amplification was initiated by denaturation for 3 min at 95 °C and 35 cycles were carried out as follows: 1 min at 95 °C, 1 min at 55 or 49 °C, and 3 min at 72 °C. The final extension was continued at 72 °C for 10 min. The amplification products were then digested with *NcoI* and *XhoI*, ligated to compatible site of pET28b(+) (Novagen, Madison, WI, USA) and transformed into *E. coli* DH5α (Stratagene, La Jolla, CA, USA) [21]. Plasmids DNA were extracted from recombinant positive clones. *E. coli* expression host BL21-CodonPlus(DE3)-RIL strains (Stratagene, La Jolla, CA, USA) were transformed with isolated recombinant plasmids and grown at 37 °C in SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl,

Table 1
Primer sequences used at amplification of Apa1 cDNA

Name	Primer	Orientation	Sequence 5′–3′	Location in cDNA (bp)	PCR product size (bp)
DNA	Apa1f	Forward	GGGGGGCC**ATG**GGCAACATTCTTCGAGGAGAGTC	0–1239	1239
Apa 1	Apa1r	Reverse	GGGGGGCTCGAGCAAATGGATTGAAATTTTGAAA		
DNA	F1f	Forward	GGGGGGCC**ATG**GGCAACATTCTTCGAGGAGAGTC	0–321	321
Fragment 1	F1r	Reverse	GGGGGGCTCGAGTTTTGAGGCGCTCACGATTCCA		
DNA	F2f	Forward	GGGGGGCC**ATG**GGCCTTGCGATCGACAAATGCGA	321–795	474
Fragment 2	F2r	Reverse	GGGGGGCTCGAGTCTGAATTGTTCCGTGTTAACA		
DNA	F3f	Forward	GGGGGGCC**ATG**GGCACATCCGATTATCAACAGAA	795–969	174
Fragment 3	F3r	Reverse	GGGGGGCTCGAGACGGATATTGTGTCTTTCAAGT		
DNA	F4f	Forward	GGGGGGCC**ATG**GGCACCGTCGCTCAAAGTGATGA	969–1239	270
Fragment 4	F4r	Reverse	GGGGGGCTCGAGCAAATGGATTGAAATTTTGAAA		

The bolded parts of primer sequences represent start codons. The underlined parts of primer sequences represent *NcoI* and *XhoI* restriction endonuclease site.

2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose) containing 30 μg/ml kanamycin to an optical density (O.D.) 0.5 at 600 nm. Isopropyl-β-thiogalactopyranoside (IPTG) was then added to a final concentration of 1.0 mM. The cells were harvested after 5 h by centrifugation (5000 rpm, 15 min at 4 °C) and stored at −20 °C until further purification.

2.4. Purification of rApa1 and its recombinant protein fragments

The rApa1 and its fragments were isolated from crude bacterial lyzate under denaturing/renaturating conditions by affinity chromatography employing Ni–NTA (Qiagen, Hilden, Germany) [22]. The re-frozen induced cells were resuspended in buffer solution 1 (6 M guanidine hydrochloride, 0.02 M Tris–HCl, 0.5 M NaCl, pH 8.0). The obtained lyzate was loaded onto the Ni–NTA column pre-equilibrated in buffer 1. Washing and solubilization steps were performed consequently with buffer 2 (6 M urea, 0.02 M Tris–HCl, 0.5 M NaCl, pH 8.0), supplemented with 0.06 M imidazole, buffer 3 (0.02 M Tris–HCl, 0.5 M NaCl, pH 8.0), and finally with buffer 4 (0.02 M Tris–HCl, 0.15 M NaCl, pH 8.0). Soluble recombinant protein and protein fragments were eluted with buffer 5 (0.02 M Tris–HCl, 0.15 M NaCl, 0.05 M EDTA, 0.5 M L-Arg, pH 8.0).

2.5. Refolding of purified recombinant proteins

The soluble recombinant protein and fragments were refolded by dialysis in dialysis bag with a MW cut-off 1 kDa over 24 h at 4 °C against renaturated buffer (0.02 M Tris–HCl, 0.05 M L-Arg, pH 8.0). During dialysis, the buffer was changed three times to entirely remove detergent.

2.6. SDS-PAGE and Western blot analysis

Electrophoresis on SDS-PAGE was carried out by the method of Laemmli [23] stained with Serva Blue (Serva, Heidelberg, Germany).

Western blot analysis of proteins was done using the semi-dry blotting method [24]. Proteins were electrophoretically transferred after SDS-PAGE to a nitrocellulose membrane. The blots were first incubated overnight in milk buffer (0.2 M NaCl, 0.05 M Tris–HCl, pH 7.4, 10% powdered non-fat milk) containing polyclonal rabbit antiserum against rApa1 at a dilution of 1:1000 and subsequently 2 h in the same buffer with peroxidase-conjugated porcine anti-rabbit IgG (SwAR Px, Institute of Sera and Vaccines, Prague, Czech Republic) at a dilution of 1:2000. Visualization of the immunoactive protein bands was performed by incubation of the membrane with 0.33% 3,3-diaminobenzidine tetrahydrochloride and 30 μg/ml hydrogen peroxide in 0.05 M Tris–HCl, pH 7.4 for 5 min.

2.7. Protein quantification

The protein content was determined using the Bradford assay [25]. Bovine serum albumin (Fermentas, Vilnius, Lithuania) was used a reference protein.

2.8. Mice

Male ICR mice aged 8 to 12 weeks were obtained from the animal farm of the Institute of Experimental Pharmacology, Slovak Academy of Sciences (Dobrá Voda, Slovakia). All animals were housed in micro-isolator cages in a temperature-controlled facility. Food and water were provided ad libitum. All animal experiments described in the article were conducted according to the guidelines by the Institute of Virology, Slovak Academy of Sciences (Bratislava, Slovakia).

2.9. Cell cultures and their activation

Peritoneal ICR murine macrophages were prepared according to Park and Rikihisha [26]. Murine peritoneal macrophages were elicited by intraperitoneal injection of 2 ml of sterile 5% thioglycolate broth (Difco Laboratories, Sparks, MD, USA). After five days, the mice were sacrificed

and peritoneal exudate cells were collected by peritoneal lavage. Cells were washed by centrifugation and 5×10^6 cells were resuspended in 0.5 ml RPMI 1640 with L-glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% heat-inactivated fetal bovine serum (FCS, Gibco BRL, Life Technologies, Karlsruhe, Germany) and placed into each well of 24-well plates. After 2 h of incubation at 37 °C in a humidified atmosphere of 5% CO_2, non-adherent cells were removed by rinsing and 0.5 ml of complete RPMI 1640 medium (10% FCS) containing the appropriate stimulants were added to each well. After cultivation, the supernatants were collected and stored at − 80 °C.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The level of TNFα was determined in cell culture supernatants collected after 3, 6, and 24 h of cultivation by ELISA kit (DUO Set, R&D systems, Abingdon, UK) according to the manufacturer's instructions. A recombinant mouse TNFα was used as a standard. The assay was repeated three times.

2.11. Stimulants of TNFα release

Native monomeric (55 kDa) form of Apa1, rApa1 with MW 48.15 kDa, rApa1-F1 (13.56 kDa), rApa1-F2 (18.79 kDa), rApa1-F3 (7.05 kDa), rApa1-F4 (11.84 kDa), and a mixture of the fragments of native Apa1 produced by using partial tryptic digestion were applied in the stimulation assay.

The stimulants were diluted to 0.9 μM final concentration in complete RPMI 1640 medium. All prepared samples were rendered sterile by filtration (0.22 μm).

2.12. Preparation of honey media for stimulation of TNFα assay

Acacia honey from Sebechleby (Slovakia) was purchased from an apiary of Mr. Peter Demian. 1% (w/v) honey solution was prepared in complete RPMI 1640 medium. The same acacia honey was deproteinized by filtration using a Microsep device (Pall Life Sciences, Ann Arbor, MI, USA) with 3 kDa cut-off. Deproteinized honey solution (30% (w/v)) was adjusted to 1% (v/v) in complete RPMI 1640 medium. All prepared honey solutions were rendered sterile by membrane filtration (0.22 μm).

2.13. Statistical analysis

Results are presented as the mean with standard deviation (SD). All data were statistically analyzed by one-way analysis of variance ANOVA and Bonferroni's test to determine whether there were differences within groups. *P* values smaller then 0.05 were considered to be significant. Analyses were performed using OriginPro 7.5 statistical software package (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Partial tryptic digestion of native Apa1

To obtain preliminarily information on digestibility of monomeric form of Apa1, its native form was obtained by size-exclusion column chromatography. The SDS-PAGE profile and Western blot analysis of partial tryptic digestion of Apa1 at different times of digestion are shown in Fig. 1. Lane 8 demonstrates the presence of three distinct protein bands after 3.5 h digestion, with apparent MW values of about 36 kDa, 27 kDa, and 15 kDa. The determined N-terminals of the protein bands were Leu-Ala-Ile-Asp, Thr-Ser-Asp-Gln-Tyr, and Thr-Val-Ala-Gln, respectively. The 36 kDa and 27 kDa distinct protein bands were already present in lane 1. All sequenced protein fragments were products of trypsin protease digestion. First amino acid before determined N-terminals was Arg, or Lys. The complete digestion of Apa1 was reached after 5 h digestion (Fig. 1, lane 10).

3.2. Expression of rApa1 and its recombinant protein fragments

The Apa1 cDNA encoding Apa1 without signal peptide sequence was subcloned into pET28b(+) vector. On the basis of N-terminal sequences of the protein bands obtained by partial tryptic digest of Apa1 (Fig. 1, lane 8), the corresponding nucleotide sequences derived from Apa1 cDNA were determined. The whole molecule of Apa1

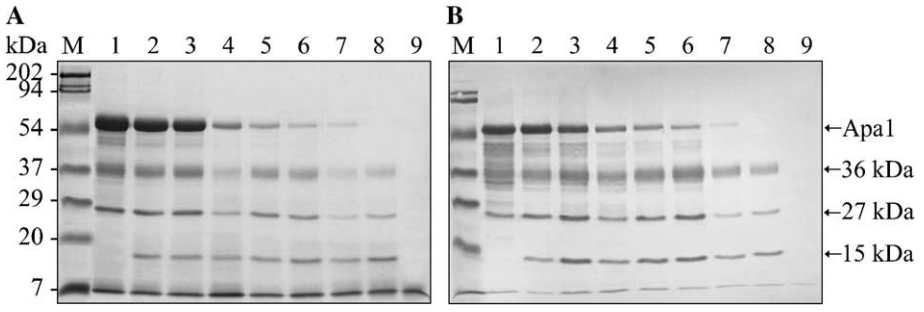

Fig. 1. Tryptic digestion of native purified Apa1. (A) SDS-PAGE (12%) analysis visualized by Serva blue staining . Lane M—prestained protein molecular weight marker (Bio-Rad), lane 1—native Apa1, lane 2—0.5 h digestion, lane 3—1 h, lane 4—1.5 h, lane 5—2 h, lane 6—2.5 h, lane 7—3 h, lane 8—3.5 h, lane 9—5 h. (B) Western blot analysis of samples as described in panel A probed with antibody against rApa1.

Table 2
Characteristics of rApa1 and its recombinant protein fragments

	MW	aar	pI
rApa1	48.15	423	5.03
rApa1-F1	13.56	117	4.85
rApa1-F2	18.79	168	4.55
rApa1-F3	7.05	68	5.38
rApa1-F4	11.84	100	5.23

The characteristics were predicted by computer analysis using Vector NTI Suite 8.0 software (InforMax, Frederick, MD, USA) of rApa1 sequence without signal peptide and of rApa-F1, rApa-F2, rApa-F3, and rApa-F4. MW: apparent molecular weight in kDa; aar: amino acid residues; pI: isoelectric point.

cDNA was thus divided into four distinct DNA fragments, designated as DNA fragment 1, 2, 3, and 4. The analysis by PCR and restriction endonuclease digestion of Apa1 cDNA and cDNA of the DNA fragments proved the correct insertion of the Apa1 cDNA and cDNA of the DNA fragments in the recombinant plasmid.

The maximum level of expression of rApa1 and its recombinant protein fragments (rApa1-F) was achieved in modified *E. coli* expression host BL21-CodonPlus(DE3)-RIL and was significantly higher compared to the expression level achieved by conventional *E. coli* BL21(DE3) cells (data not shown).

3.3. Purification of rApa1 and its recombinant protein fragments

The expressed rApa1 and its recombinant protein fragments contained three additional amino acid residues and cluster of six histidine residues for purification by metal-affinity chromatography. Molecular characteristics of recombinant proteins are shown in Table 2.

The purity of rApa1 and its protein fragments were examined by SDS-PAGE (Fig. 2A) and positively identified by immunoblotting with polyclonal rabbit antiserum against rApa1 (Fig. 2B). All expressed recombinant proteins were

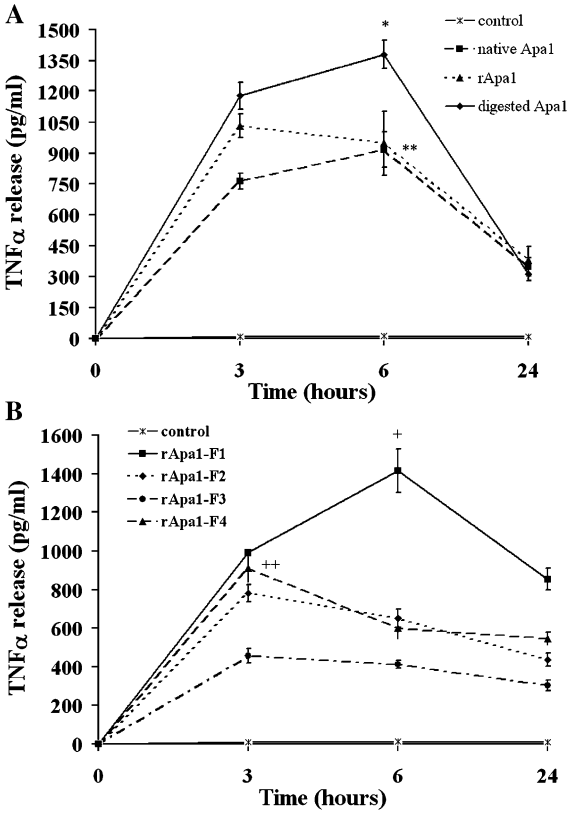

Fig. 3. Time dependence of the effect of the various forms of Apa1 (A) and recombinant protein fragments (B) (at 0.9 μM) on stimulation of TNFα secretion by murine peritoneal macrophages measured after 3, 6, and 24 h of cell cultivation. Data are expressed as mean with SD from at least three independent experiments analyzed by ANOVA and Benferroni's pair-wise tests. $^{*}P<0.001$ was calculated vs. digested Apa1 after 24 h, and vs. native Apa1 and rApa1; $^{*}P<0.05$ vs. digested Apa1 after 3 h; $^{**}P<0.001$ vs. native Apa1 after 24 h; $^{**}P<0.05$ vs. native Apa1 after 3 h. $^{+}P<0.001$ was calculated vs. rApa1-F1 after 3 and 24 h; $^{+}P<0.001$ vs. native Apa1 after 24 h.

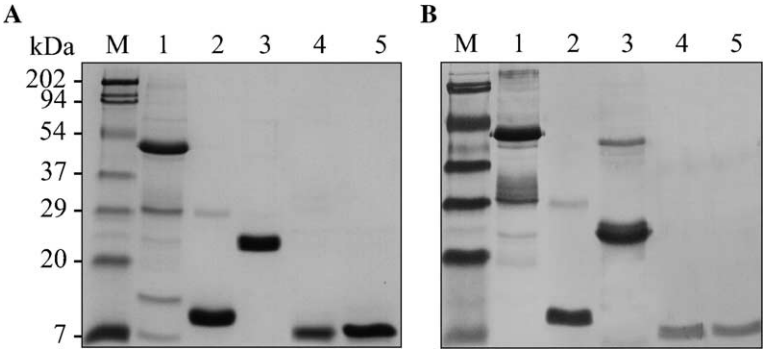

Fig. 2. Recombinant Apa1 and its recombinant protein fragments. (A) SDS-PAGE (12%) analysis of recombinant proteins after dialysis against 50 mM L-Arg visualized by Serva blue staining. Lane M—prestained protein molecular weight marker (Bio-Rad), lane 1—rApa1, lane 2—rApa1-F1, lane 3—rApa1-F2, lane 4—rApa1-F3 and lane 5—rApa1-F4. (B) Western blot analysis of samples as described in panel A probed with antibody against rApa1.

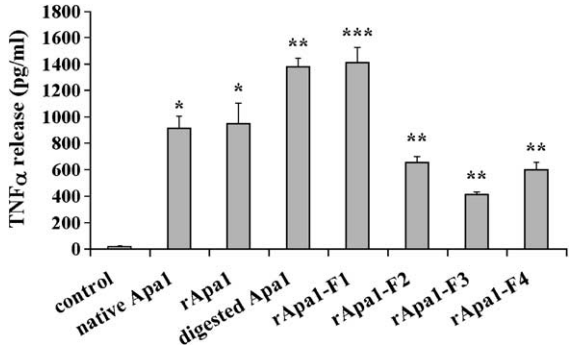

Fig. 4. Comparison between the protein (at 0.9 μM) stimulation of TNFα secretion by murine peritoneal macrophages measured after 6 h of cell cultivation. Data are expressed as mean with SD from at least three independent experiments analyzed by ANOVA and Benferroni's pair-wise tests. ***$P<0.001$ was calculated vs. other recombinant protein fragments and native or recombinant Apa1, **$P<0.001$ vs. non-stimulated cells (control), and *$P<0.05$ vs. rApa1-F2, rApa-F3, and rApa1-F4.

present in insoluble cytoplasmic fraction as the inclusion bodies (data not shown). Recombinant proteins were purified by a single-step purification/renaturation procedure on a nickel-nitrolotriacetic acid–agarose resin (Ni–NTA) column. Elution buffer was supplemented with 0.5 M L-Arg that improves solubility of recombinant proteins and assists at their refolding.

Lane 1 in Fig. 2B revealed besides refolded rApa1 presence of traces of several degradation products that were immunoactive against polyclonal rabbit antiserum of rApa1. The SDS-PAGE showed that MW values of all recombinant proteins corresponded to the calculated values with the exception of rApa1-F2. The migration profile of rApa1-F2 in SDS-PAGE (Fig. 2A, lane 3) indicated that MW (23 kDa) of the protein was not in agreement with a predicted MW (18.79 kDa), which could have been caused by specific characteristics of the protein. The minor bands migrating at 28 kDa (lane 2) and 46 kDa (lane 3) in Fig. 2B represented dimeric forms of rApa1-F1 and rApa1-F2, respectively.

3.4. Effect of native and recombinant Apa1 on TNFα production by murine macrophages

In this study, we used all immunostimulators at 0.9 μM concentration, which corresponded to 50 μg/ml of Apa1, in order to compare their immunostimulatory effect on TNFα release. The maximum effect of TNFα release by native Apa1 was noted at 0.9 μM (50 μg/ml) concentration and higher concentration of Apa1 did not increase the level of TNFα release (data not shown).

All stimulants caused augmented release of TNFα from murine peritoneal macrophages when compared with the untreated cells (Fig. 3). The TNFα production by macrophages was measured after 3, 6, and 24 h. TNFα production increased in time, reaching a maximum value between 3 and 6 h of cultivation. Extension of the cultivation to 24 h did not result in elevated cytokine secretion with all tested stimulants (Fig. 3).

Six hours after stimulation with the native Apa1 and rApa1, TNFα production reached a maximum level 917.9 ± 85.6 and 948.9 ± 154.3 pg/ml, respectively (Fig. 4). The difference in TNFα release between these stimulants was not statistically significant. The native Apa1 subjected to a limited tryptic digestion for 3.5 h elicited significant secretion ($P<0.001$) of TNFα with a maximum value of 1379.0 ± 68.7 pg/ml reached upon 6 h of stimulation.

All recombinant protein fragments differently affected the production of TNFα by macrophages. Application of the renaturated buffer (without any proteins) induced only a baseline level of TNFα production, which was the same as that observed with the untreated cells (data not shown). Administration of rApa1-F1 led to a significantly ($P<0.001$) higher TNFα release than the other protein fragments and

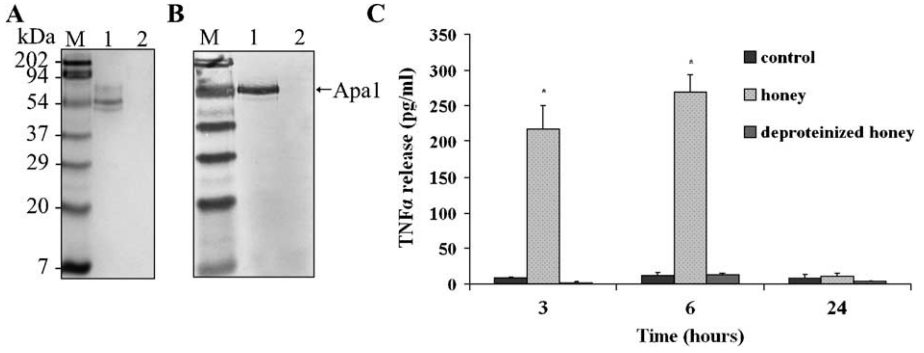

Fig. 5. Detection of the Apa1 in honey and its effect on TNFα release. (A) SDS-PAGE (12%) analysis of proteins in 30% (w/v) honey samples visualized by Serva blue staining. Lane M—prestained protein molecular weight marker (Bio-Rad), lane 1—honey, lane 2—pretreated honey without proteins. (B) Western blot analysis of the samples as described in panel A probed with antibody against rApa1. (C) Time dependence of the effect of 1% (w/v) honey and of the deproteinized honey on TNFα secretion by murine peritoneal macrophages measured after 3, 6, and 24 h of cell cultivation. Data are expressed as mean with SD from at least three independent experiments analyzed by ANOVA and Benferroni's pair-wise tests. *$P<0.001$ was calculated vs. non-stimulated cells (control) and deproteinized honey.

rApa1 after 6 h stimulation, yielding a maximum value of 1414.8 ± 112.2 pg/ml (Fig. 4). The differences in the immunostimulatory effects between rApa1-F1 and native or recombinant Apa1 were statistically significant ($P<0.001$). Furthermore, the immunostimulatory effect of rApa-F1 was significantly higher ($P<0.001$) after 6 h of cultivation than after 3 and 24 h (Fig. 3B). No significant difference was found between stimulatory capacities of digested native Apa1 and rApa-F1.

3.5. Effect of honey on TNFα production by murine macrophages

Honey at concentration 1% (w/v) was compared with honey that contained no proteins for stimulation of production of TNFα. SDS-PAGE and immunoblotting profile of 30% (w/v) honey's samples are shown in Fig. 5A,B. The most intensive band corresponds to a 55 kDa protein with mobility identical to that of Apa1 (Fig. 5A, lane 1). The identity of Apa1 in honey was confirmed by Western blot analysis using polyclonal antibodies raised against rApa1 (Fig. 5B, lane 1). Honey significantly stimulated production of TNFα (269.5 ± 23.7 pg/ml) in comparison to the deproteinized honey and to untreated cells, after 6 h of stimulation ($P<0.001$) (Fig. 5C). Deproteinized honey stimulated only 13.4 ± 1.3 pg/ml of TNFα release after 6 h of stimulation.

4. Discussion

The broad spectrum of physiological activity of the honeybee proteins may be demonstrated on proteins of larval diet, particularly proteins of RJ. The new experimental knowledge on the similarities between the immune systems of insects and mammals indirectly confirms the original empirical observations that RJ can play a role of an immunostimulator [27,28]. The recent discovery that RJ-proteins may have physiological functions as suppressors of allergic reactions, as well as their established anti-hypertensive and proliferation stimulatory properties, opened a new era in application of RJ and honey [3,13,16,17].

Many peptides identified in the natural sources are known for their non-specific immunostimulatory responses [29]. However little is known about the immunomodulatory proteins. The peptide and protein immunomodulators, in general, generate a physiological response in the target cells via their specific surface receptors. Transcription factor NF-κB is a major activator for TNFα transcription in macrophages and regulates TNFα transcription in response to wheat gliadin immunomodulator [30]. Nevertheless, in most cases the process, by which production of these immunomodulators is triggered in the organism, is still unclear.

For monitoring immunomodulating activity of the novel potential immunomodulators, macrophages appear to be a suitable model. Stimulation of macrophages leads to gradual changes in their properties and only activated macrophages secrete cytokines (TNFα, IL-1 and IL-6).

TNFα has been shown to play an essential role during the inflammatory response, exerting dual, both beneficial and deleterious, effects [31]. TNFα is a key cytokine involved in antiviral, antibacterial, and antiparasitic host defense mechanisms [32,33]. Due to their proven TNFα eliciting activity, small amounts of immunomodulators are believed to be of importance for the development and maintenance of vital physiological functions of the host.

For determination of the immunostimulatory activity of recombinant proteins, it was important to prepare pure, soluble, and stable samples of the concentrated proteins. A number of strategies have therefore been developed to improve the yield of soluble renaturated proteins [34–36].

Sequential analysis of Apa1 showed that it contained high content of essential amino acids Arg, Leu, and Ile [5]. Apa1 is enriched with codons namely AGG/AGA (Arg) and CGA (Arg) that are rarely used by *E. coli* and may not be expressed efficiently in conventional *E. coli* cells. Previously, we attempted to perform the heterologous expression of rApa1 in pQE32 system in *E. coli*, however, without achieving a sufficient expression level and with limited protein solubility [37]. The low expression level of Apa 2 in pQE32 system in *E. coli* [38] was probably caused also by the mentioned limited arginin codon usage by *E. coli*.

The similar situation was observed also with tobacco, where rApa1 expression was analyzed in transgenic plants [39]. Only weak expression of several glycosylated forms of rApa1 was detected in the plant. Larger quantity of soluble rApa1 and its recombinant fragments were obtained using pET28b(+) system in modified *E. coli* BL21-CodonPlus(DE3)-RIL with extra copies of *E. coli* argU, ileY and leuW tRNA genes. Preparation of the concentrated and long-term stable recombinant protein samples is required using single additive positively charged L-Arg. Here, we demonstrated that the presence of 50 mM L-Arg in concentrated protein solutions significantly reduced aggregation or precipitation during purification and dialysis. The effect of L-Arg on the solubility and aggregation is dependent on the protein charge and amino acid concentration [36]. At lower concentrations (<0.1 M), L-Arg causes preferential hydration of proteins, thus, stabilizing them [40] and it serves as an

agent that preferentially destabilizes the incorrectly folded proteins. However, little is known about the mechanism [41].

The molecule of rApa1, in contrary to all recombinant protein fragments, was partially degraded. Probably, such proteolytic degradation has already been initiated during the expression of protein in the cells *E. coli*. We assume that recombinant protein fragments do not contain specific sequences, which are necessary for proteolytic degradation by a complex of proteases from *E. coli*, particularly trypsin-like proteases. Moreover, the degradation of native monomeric, but not oligomeric, form of Apa1 in RJ was also observed [19,42–44]. It has been recently indicated that Apa1 in RJ is specifically degraded by unknown metalloproteinase(s), which seem to have restricted substrate specificity [43]. On the other hand, RJ has been reported to contain trypsin-like protease [45], which could be involved in the specific degradation of Apa1.

A number of previous reports have indicated that Apa1 is involved in various biological events. As a result of tryptic proteolysis, Apa1 has no stimulatory effect on hepatocyte DNA synthesis [42] but increases its immunostimulatory effect on production of TNFα by murine macrophages. Moreover, all recombinant protein/fragments, particularly rApa1-F1, had the stimulatory effects on the production of TNFα by macrophages. It is possible that there are uncovered domain(s) on rApa1-F1, which contain specific sequences involved in an immunostimulatory effect. Extension of the cultivation to 24 h resulted in reduced TNFα secretion with all tested stimulants. We suppose that the decreased TNFα concentration measured after 24 h resulted from the proteolytic degradation of TNFα.

It has been found that N-linked glycoprotein Apa1 bears a typical high-mannose type oligosaccharide as major component of N-glycans [46]. Until now it has not been known which part(s) of glycoprotein Apa1 are responsible for stimulation of TNFα production. In this report we have demonstrated that only amino acids moiety of Apa1 is responsible for the release of TNFα from murine macrophages. Native glycoprotein Apa1 had comparable capacity to stimulate murine TNFα as rApa1 prepared by heterologous expression in *E. coli*. Presence or absence of N-linked glycans in Apa1 did not affect the immunostimulatory activity (Fig. 3). These observations are consistent with the recently published report that 70 kDa protein Apa3, but not N-linked oligosaccharide on Apa3, contributes to IL-4, IL-2, and INF-γ production–suppressive activity exhibited by Apa3 [3]. It is possible that the oligosaccharide on Apa3 may be of the high-mannose type, which is often produced by insect cells [3]. Therefore, we suppose that carbohydrate moiety of major RJ-glycoproteins is not important for their cytokine production or suppressive activity. On the other side, it is known that glycosylation of proteins affects their biological functions, such as protein stability, protein secretion, receptor interaction, and subsequent downstream biological activity. It is possible that physiological function of a carbohydrate component on Apa1 involves mechanisms not apparent in vitro.

Apa1 was immunochemically identified in honey and in honeybee pollen as a dominant protein [4]. At least 19 protein bands were detected by silver-staining SDS-PAGE in honeys of different plant origins [47]. Recently, it has been discovered that 1% honey stimulates release of TNFα in human monocytic cell line, though artificial honey (mixture of glucose and fructose similar to that found in natural honeys) failed to elicit TNFα release [14]. We demonstrate that protein content of honey is responsible for activation of macrophages. The main contribution to stimulation of TNFα can be probably attributed to a dominant protein Apa1. Other proteins of honey are present in significantly at lower concentration than Apa1 but until now, no quantitative determination of Apa1 in honey has been carried out.

Apa1 may also play a role in honeybee immunity. Induction of innate immunity in mammals and insects leads to the activation of similar effector mechanisms [48]. Furthermore, cytokine-like activity of certain molecules has been increasingly described in some invertebrates, including insects [49–51]. Drosophila genome encodes a TNFα-like molecule and a receptor with a TNFR-like extracellular domain. However, this receptor does not contain a cytoplasmic domain homologous to either TNFR [49]. The role of these TNF-like factors in insect immunity remains unknown.

In conclusion, our results document that proteinous part of glycoprotein induces TNFα production from murine peritoneal macrophages. This immunostimulatory effect is significantly increased even after a limited proteolytic digestion. We have found that N-terminal region of molecule Apa1 markedly elicits release of TNFα. Moreover, the data from our ongoing experiments show that the stimulatory effect of honey with regard to the production of TNFα is based on its protein content, primarily on its dominant protein Apa1.

Further studies are needed for investigation of the molecular mechanisms of immunostimulatory effect of Apa1 and its oligopeptides as antimicrobial defensive barrier during larval development of honeybee.

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