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ANTIMICROBIAL AND ANTI-INFLAMMATORY ACTIVITY OF CAMEL MILK DERIVED IMMUNE PROTEINS AND PEPTIDES AGAINST *PROPIONIBACTERIUM ACNES*

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ABSTRACT

It has been long described the major role of *Propionibacterium acnes* in the pathogenesis of acne vulgaris disease. There is increasing evidence that particularly persistent, relapsing and difficult-to-treat infections caused by *P. acnes* are associated with the emergence of the drug resistant strains. The aim of this study is to evaluate the potential antimicrobial effects of Camel milk derived antimicrobial proteins and peptides against *P. acnes* by micro broth dilution assay. Here, we show that peptidoglycan recognition proteins PGRPs possess the strongest antimicrobial activity against *P. acnes* as compared to Lactoferrin and polyclonal anti-*P. acnes* antibodies. In addition, significant anti-inflammatory activity of PGRPs, and Lactoferrin was reported; however, less than the *Anti-P. acnes* antibody fraction. In conclusion, Camel derived immune

proteins and peptides exert potent antimicrobial activity against *P. acnes* and could be considered as supportive and potent options in the treatment regimen of Acne vulgaris.

Key words; Acne vulgaris, Innate immune proteins, Antimicrobial peptides, PGRP, and Anti-inflammatory effects

INTRODUCTION

Acne vulgaris is a major skin disease affecting young adults worldwide. The disease affects mostly the sebaceous glands leading to characteristic inflammatory lesions called comedones in the face, back, and trunk. The presence of obligated anaerobic Gram positive bacterium *Propionibacterium acnes* (*P. acnes*) in the follicular and sebum canals has essentially involved in the development of inflammatory acne [1]. *P. acnes* is capable to interact with the components of the immune system and bio-metabolize sebum fats into free fatty acids, leading to a remarkable inflammatory response presented with chemotaxis of neutrophils and induction of monocytes to produce various pro-inflammatory mediators including tumor necrosis factor (TNF) and Interleukin -8 (IL-8) [2]. Topical therapy is inevitable in acne treatment and is mainly applied for the mild cases while in more severe forms, a combined topical and systemic therapy is routinely used. Due to both direct and indirect effects on the patho-genetic factors and the severity of the acne lesions, therapeutic success in acne and related skin disorders is not always ensured [3]. Major drawbacks associated with the commonly used topical agents for the treatment of acne include, but not limited to, the patient compliance since the regimen usually last for long periods. Antibiotics and isotretinoin constitutes first line of treatment options of Acne. However, development of resistance by bacteria in addition to teratogenic effects of isotretinoin, would drive toward the investigation for more potent and safer therapeutic options [4].

Recent interest in the use of antimicrobial peptides (AMPs) and proteins of the innate immunity as therapeutic adjunct has emerged and been thoroughly investigated. Several studies showed superior *in vitro* antimicrobial effects of various proteins and AMPs against a wide variety of both Gram positive and Gram negative bacteria [5, 6]. However, human derived immune proteins and AMPs have limited supply and less stability at room temperatures rendering them not much useful candidates for therapeutic agents. On the contrary, Camel milk derived agents showed satisfying competencies

among other products used for different applications[7]. Camel milk has highly rich of unsaturated fatty acids with anti-cancer, hypo-allergic and anti-diabetic properties[8]. Other components such as peptidoglycan recognition proteins (PGRPs), Lactoferrin, immunoglobulins, lysozyme, and vitamin C were reported to contribute to these properties [9, 10]. For example; camelids Lactoferrin and antibodies showed outstanding physical characteristics in terms of solubility and stability as proved by remained functionally active, even after exposure to harsh conditions, such as heat and pH variations[11].

In this context, this study aims to evaluate the antimicrobial and anti-inflammatory effects of camel milk derived antimicrobial peptides and antibodies against *P. acnes*.

MATERIALS AND METHODS

Microbiological and cellular assays

Bacterial strains and growth conditions

A standard strain of *P. acnes* (NCTC 747), kindly provided by the Jordan Company for Antibody Production (Monojo), was used in this study. Standard bacterium inoculum of 1.0×10^8 CFU / mL was prepared in tryptic soy broth supplemented with 1% glucose. Cultures were incubated at 37^{o}C for 48 h under anaerobic conditions using CampyGen atmosphere generating system (Oxoid, UK). Subcultures were made on agar base constituted of molten TSA with glucose.

Antimicrobial activity of Immune proteins and AMPs against P. acnes

In the standard microdilution method, the antimicrobial activity of camelid PGRP, Lactoferrin and polyclonal antibodies with specificity to *P. acnes* was evaluated. Serial dilutions of each type of the protein preparations (0.05 to 1 mg / mL) were incubated with the bacteria and for 5 h in sodium phosphate buffer (pH 7.2) containing 1% trypticase soy broth and glucose. The remaining bacteria were determined by plating on TSA with glucose (Oxoid, UK) agar plates and counting colony-forming units after incubation at 37^{o}C for up to 3 days [12].

Measurement of pro-inflammatory cytokines

Heat killed *P. acnes* preparation from log-phase bacterial culture was used for induction of inflammation. In brief, an initial inoculum of *P. acnes* cultured in BHI broth with 1% glucose for 72 h at37^{o}C under an anaerobic condition was harvested, washed three times with PBS, and incubated at80^{o}C for 30 min to heat inactivate and kill the bacteria. Seeded human monocytic THP-1 cells (Thermo Fisher Scientific, UK) at 1 x 10^6 cells/ml in 24-well plates with serum-free

medium, and were stimulated with 0.1 mL of heat-killed *P. acne*s (inflammation control) and with 0.1 mL of premix solution of heat killed *P. acnes* in combination with different concentrations (0.01 to1 mg/mL) of tested protein fractions for an 24 h incubation. Cell-free supernatants were collected, and concentrations of TNFα, IL-1α, and IL-8 (Invitrogen, UK) were analyzed with respective enzyme immunoassay kits.

Camel Milk Proteins isolation, measurements and characterization

Camel milk collection and protein preparations

A total amount of 10 Liters of camel milk enriched with antibodies against *P. acnes* (NCTC 737) were collected. Lipids removal was carried out by centrifugation at 15,600 xg/0◦C for one hour (Hermile, Germany). Removal of Casein was achieved by adding 100 mg/L Rennet (Valiren, USA). The generated liquid phase whey was collected, sterilized and lyophilized (zirbus freeze dryer, Germany) [13].

Isolation of Immune proteins and AMPs

Lactoferrin was isolated from Camel milk as described previously [14]. PGRP proteins were purified from whey isolate by nickel-agarose (His-Bind Kit, Novagen) affinity chromatography under native conditions following dialysis as described by Kappeler *et al*., 2004[15]. Buffers included 4mM $CaCl_2$ and 10% glycerol, and elution was done with 300 mM imidazole. PGLYRP-containing fractions were dialyzed against 10mMTris, pH7.6, with150mMNaCl, 5mM$CaCl_2$, and 10% glycerol. Fractionation of the whey proteins was performed by cation-exchange fast protein liquid chromatography (FPLC; Amersham Pharmacia, Uppsala, Sweden) on a Protein Pak SP 5PW column (7.5 mm × 75 mm; Waters) Milford, MA, USA). Imidazole-hydrochloric acid 0.01 M pH 7.0 was used as the equilibration buffer. A total of 500 ul proteins (5 mg/mL) were loaded into the column, and proteins were eluted with a 0–1 M NaCl gradient from 5 to 30 min at a flow rate of 1 ml/min. Detection was performed at wavelength of 280 nm.

Measurements of Anti-P. acnes Antibodies in camel whey

Specific antibodies against *P. acnes*were measured by indirect Enzyme-Linked Immunosorbent Assay (ELISA). In brief, flat bottomed 96 well polystyrene micro titer plates (Greiner, Germany) were coated with 100 µl of 10 µg/ml *P. acnes* antigens in carbonate-bicarbonate buffer (pH 9.6) overnight at 4° C[16].Subsequently, three time washing of the plates were performed with 100 µl of 0.15 M PBS (pH 7.2)

containing 0.05% Tween 20 and then plates were blocked with 200 µl of 2% bovine serum albumin (BSA) in PBS. A total of 100 µl of the immunoglobulin fraction isolated from whey protein preparations samples diluted at 1:100 in 1% BSA/PBS were added in duplicates and incubated for one hour at RT. Negative and positive control samples were included in each run. Enzymatic detection system made of horse radish peroxidase (HRP) conjugated protein A and protein G diluted at 1:1000 in 1% BSA/PBS and 0.1% O-phenylenediamine (Sigma, USA) containing hydrogen peroxide in 0.1 M citrate buffer (pH 4.5). The absorbance was measured at 490 nm using ELISA reader (AsysHitech, Switzerland).

Characterization of AMPs and Proteins Using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoreses (SDS-PAGE)

Components of camel whey protein isolates were separated by the standard SDS-PAGE described by Ahmad *et al*., 2018 [16]. In brief, 0.5 mm thick 10% acrylamide-bisacrylamide gels under non-reducing conditions as were used. Resolving gels of 4 mL distilled water, 2.5 mL running buffer (pH 8.8), 3.3 mL of 30% acrylamide-bisacrylamide solution, 100 µL of 10% ammonium persulfate (APS) and 10 µL Tetramethylethylenediamine (TEMED) were firstly prepared. Stacking gels (4%) were prepared by adding 6 mL of distilled water, 2.5 mL staking buffer (pH 6.6), 1.3 mL of 30% acrylamide-bisacrylamide solution, 100 µl of 10% Ammonium persulfate (APS), and 10 µl TEMED. Protein fractions were mixed with an equal volume of sample buffer lacking β-mercaptoethanol (non-reducing conditions) (pH 6.8). Molecular weight protein standard was used for estimation of band sizes. Electrophoresis conditions included running buffer with pH 8.3, 120 volts and time of 60-120 min. The gels were visualized after staining with 0.2 % Coomassie brilliant blue R-250 and washing with 20% acetic acid until clear bands were seen.

RESULTS AND DISCUSSION

The aim of this study was to evaluate the antimicrobial and anti-inflammatory activity of Camel milk derived antimicrobial peptides (AMPs). To this end, we analyzed the activity of Lactoferrin, peptidoglycan recognition proteins (PGRPs) and immunoglobulins specific to *P. acnes* isolated from milk of lactating Camels previously used for production of enriched whey [13]. These AMPs were selected because of their abundancy (figure 1), stability and known superior physiochemical

and biological activities making them excellent potential for use in prophylactic and therapeutic cosmetic formula [16].

The antimicrobial effect of the tested protein factions by the microdilution assay revealed highest activity of PGRPs and Lactoferrin against *P. acnes* (Table 1). Anti-*P. acnes* specific antibodies did not show growth inhibitory or bactericidal activity against *P. acnes*.

It was clear that PGRPs Inhibited the growth of *P. acnes* more efficiently than other protein fractions isolated from Camel milk even when compared to the total whey protein isolate. To further investigate whether these selected AMPs and protein fractions possess biological properties against inflammatory acne, analysis of their inhibitory effects on the pro-inflammatory mediator secretion in co-culture of THP-1 cells with heat-killed *P. acnes* was conducted. It was previously described that *P. acnes* is a major factor that is involved in the inflammatory nature of acne since the ability of this micro-organism to metabolize fatty acids in the sebum and the production of extra-cellular toxins like CAMP factor would induce monocytes to secrete pro-inflammatory cytokines especially TNF-α, IL-1α, and IL-8 [17]. We performed an ELISA for TNF-α, IL-1α, and IL-8, in supernatants THP-1 monocytes of treated with heat killed *P. acnes*. As shown in figure 2, the specific antibodies against *P. acnes* reduced significantly the release of the pro-inflammatory mediators specially TNF-α and IL-8. In contrast, PGRPs and Lactoferrin were inferior as inhibitors to the release of these pro-inflammatory mediators and they would have no major role in the management of inflammatory acne particularly if they will be used singly not in combination based therapeutic regimens. Interestingly, the best anti-inflammatory effect was seen in the whey protein isolate dictating the presence of synergism or adding effect of other AMPs or even small molecules in Camel milk. TNFα is a multi-effector cytokine produced mainly by activated macrophages. IL-8 is a major cytokine in the inflammatory acne process in which it works as a chemotactic factor, which promotes attraction of neutrophils to the pilosebaceous unit [18].

Several studies have addressed the unique biological activities of camel milk in the management of different infections and diseases. In regards to the antimicrobial activity of Camel milk, potent anti-Staphylococcal and anti-listeriosis activities of colostrum collected from camel milk were reported [19, 20]. Moreover, Anti-inflammatory and anti-cancer activities of

Camel milk have been reported [21, 22]. However, the underlying mechanisms and the major factors responsible for these biological effects are still under investigation by many researchers. Here, we report for the first time the anti-acne potential of PGRPs isolated from Camel milk with a modest anti-inflammatory activity. The benefit of camelid PGRPs is their long term stability and wide spectrum of activity. PGRPs are part of the pathogen associated molecular pattern (PAMPs) molecules which are essential components of the innate immune system [23]. According to Sharma *et al.,* 2011, camelid PGRPs have distinct structural moiety compared to human PGRPs and these moieties allow camelid PGRPs to bind to LPS of Gram negative bacteria, Lipoteichoic acid of Gram positive bacteria in addition to the peptidoglycan layer of bacteria [24]. Few reports have shown anti-inflammatory effects of human PGRPs. In a study conducted by Park *et al.,* 2011, PGRPs were expressed in the skin of mice, modulated sensitivity to experimentally-induced atopic dermatitis and contact dermatitis [25]

Camel antibodies are well characterized in the literature for their unique stability and maintenance of the biological activity even after degradation[26] In this study, Camelid antibodies showed potent anti-inflammatory effect with reduction of most of the pro-inflammatory mediators with concentrations of 0.2 mg / ml. The exact mechanism of this novel anti-inflammatory activity of the antibodies could not be completely explained however, it could be postulated that soluble factors in the head killed *P. acnes* preparation might have a role in acne mediated inflammatory response and a fraction of the generated antibodies from the immunized camels were produced to these inflammatory factors. Further work is need to characterize this observation.

Table 1: Growth inhibitory effects of PGRPs, Lactoferrin and Anti-P. acnes antibodies isolated from Camel milk against *P. acnes* (ID_{90}; Inhibitory concentration need to inhibit the growth of 90% of the bacteria, NE: no effect)

Protein preparation	ID_{90} (mg / mL)
PGRPs	0.2
Lactoferrin	0.8
Antibodies	NE
Whey Protein Isolate	0.8

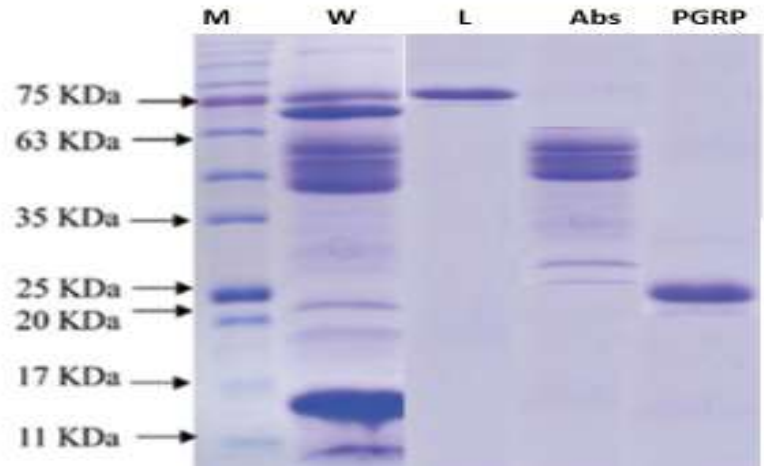

Fig 1: SDS-PAGE profile of AMPs and Protein fractions from Camel milk. M = protein marker, W= whey proteins, L=Lactoferrin fraction, Abs= Antibodies purified by proteins G/A and PGRP indicates the PGRP fraction

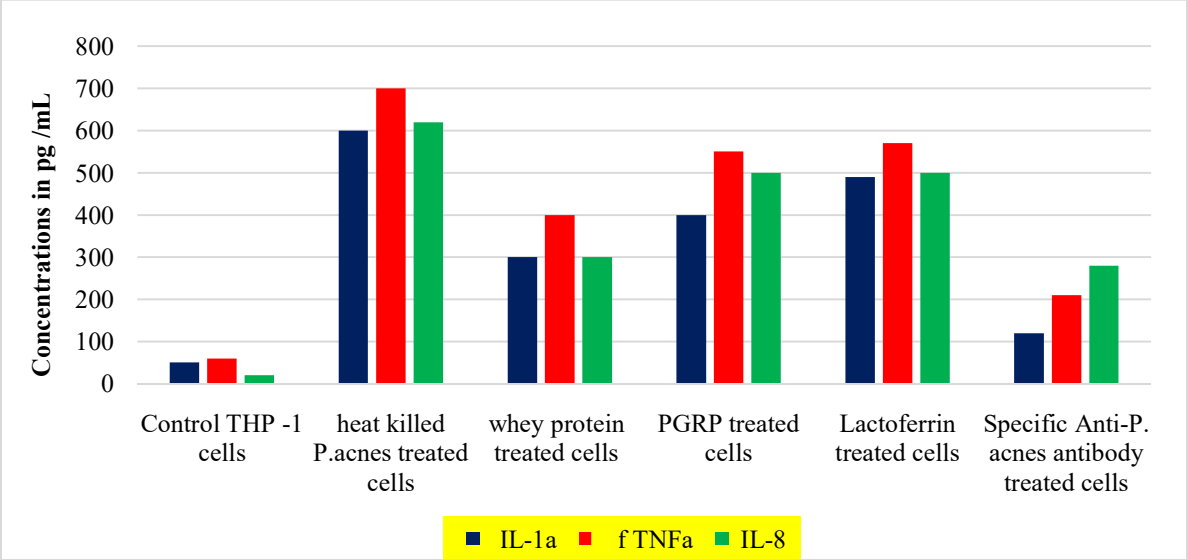

Fig2: Effects of Immune Protein fractions of Camel milk on the release of pro inflammatory cytokines TNF-α, IL-8 and IL-1α in heat-killed Propionibacterium acnes-treated THP-1 cells. Control was made of THP-1 not treated with heat-killed *P. acnes*

CONCLUSION

In conclusion, AMPs and proteins collected from Camel milk have considerable antimicrobial and anti-inflammatory effects on *P. acnes* and would provide new trend toward applying these biological molecules in the management of acne vulgaris and other skin related infections.

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