Plant protease enzymes improve wound inflammation by modifying macrophage function

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ABSTRACT

BACKGROUND: Endogenous proteases play critical physiological roles in inflammation. Plant protease enzymes (PPE) are extensively used in wound care as topical ointment or debridement agent. The objective of this study was to test the effect of PPE Ficin, Actinidin and Bromelain on macrophage function with emphasis on wound inflammation. **METHODS:** THP-1 human monocytes were differentiated to macrophages with PMA (20ng/ml, 48 h). The differentiated macrophages were treated or not (vehicle or protease inhibitor cocktail PIC) with Ficin (10^{-6} % v/v), Actinidin (5 X 10^{-4} % v/v) or Bromelain (10^{-5} % v/v), 24 h). Treated macrophages were analyzed for phagocytosis, efferocytosis, basal and LPS-induced intracellular pro-inflammatory (IL-1β) and anti-inflammatory (IL-10) cytokine levels. Murine wound macrophages were isolated using CD11b magnetic beads. Circular sterile PVA sponges (8 mm Θ) were subcutaneously implanted on the back of adult C57BL/6 mice. Sponges were harvested in early (day 3) and late (day 10) inflammatory phases. Harvested wound macrophages were also treated with PPE and analyzed for cytokine levels. **RESULTS:** For THP-1 differentiated macrophages, PPE treatment improved phagocytosis ($p<0.05$; n=5). PPE treatment boosted efferocytosis in THP-1 differentiated macrophages ($p<0.05$; n=6). Ficin as well as Actinidin potentiated the expression of anti-inflammatory cytokine (IL-10) ($p<0.05$; n=4). For wound macrophages, following d3 of PPE treatment, intracellular pro-inflammatory cytokine IL-1β was potentiated ($p<0.05$; n=4). On d10 (late phase) of PPE treatment, Ficin and Bromelain potentiated the expression of anti-inflammatory cytokine IL-10 and Actinidin downregulated the expression of IL-1β ($p<0.05$; n=4). The effects of PPE were blunted or eliminated in the presence of PIC demonstrating direct effect of protease activity. **CONCLUSIONS:** This work presents maiden evidence that topical application of PPE is capable of modifying wound inflammation by virtue of its protease activity.

BACKGROUND

- Wound inflammation is regulated by multiple factors including extracellular matrix (ECM) rich wound environment.
- Macrophages play a key role in wound healing and tissue repair by secretion of cytokines and chemokines that regulates the inflammatory process.
- The timely resolution of acute inflammation is essential to proper healing. Such resolution of inflammation is accomplished by the transition of macrophage phenotype from inflammatory type to reparative one. The non-resolving persistent inflammation leads to wound chronicity.
- Proteases play key role in numerous aspects of health and disease.
- Skin renewal, desquamation or cell shedding in mammalian skin, is regulated by these proteases.
- Plant cysteine proteases have well characterized botanical function and are of value to a wide range of therapeutic purposes.
- The objective of this study was to test the effect of PPE Ficin, Actinidin and Bromelain on macrophage function with emphasis on wound inflammation.

OBJECTIVE

Understand the mechanism of action of plant protease enzymes Ficin, Actinidin and Bromelain on wound macrophage function

METHODS

- **PPE impact on immune responses *in vitro*:** THP-1 differentiated macrophages model: THP-1 human monocytes were differentiated to macrophages with PMA (20ng/ml, 48 h). The differentiated macrophages were treated or not (vehicle or protease inhibitor cocktail PIC)
- **PPE impact on immune responses *ex vivo*:** Murine model: C57bl/6 mice. Polyvinyl alcohol (PVA) sponges were subcutaneously implanted and wound macrophages (CD 11b+) were harvested on d3 and d10 post implantation

RESULTS

PPE induced efferocytosis in human cultured macrophages

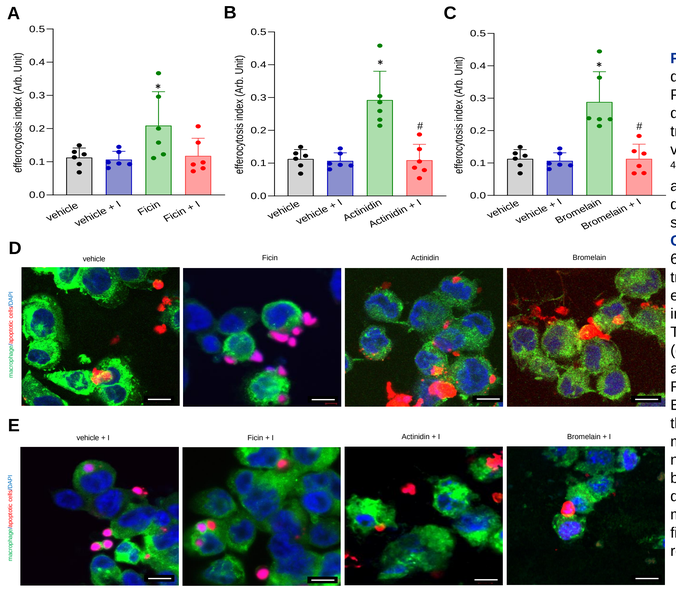

Figure 1. THP-1 cells were differentiated to macrophage with PMA (20 ng/ml, 48 h). The differentiated cells were then treated with PPE (vehicle gel 1 % v/v, ficin 10^{-6} % v/v, actinidin 5 X 10^{-4} % v/v and bromelain 10^{-5} % v/v) and PIC (0.3 % v/v) THP-1 differentiated macrophages were subjected to efferocytosis assay (**A-C**). Data represent mean ± SD (n = 6). *p<0.05 compared to vehicle-treated and #p<0.05 compared to enzyme-treated. Representative images showing efferocytosis by THP-1 differentiated macrophages (green, F4/80) cultured with apoptotic thymocytes (red, pHrodo Red succinimidyl ester) (**D, E**). Efferocytosis index of apoptotic thymocytes engulfed by macrophages, calculated as total number of apoptotic cells engulfed by macrophages in a field of view divided by total number of macrophage present in the same field of view. Scale bars in images represent 100 µm.

PPE induced phagocytosis in human cultured macrophages

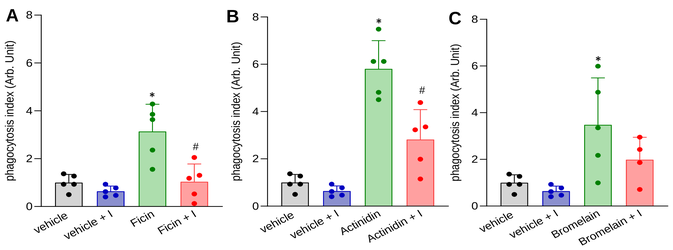

Figure 2. THP-1 cells were differentiated to macrophage with PMA (20 ng/ml, 48 h). The differentiated cells were then treated with PPE (vehicle gel 1 % v/v, ficin 10^{-6} % v/v, actinidin 5 X 10^{-4} % v/v and bromelain 10^{-5} % v/v) and PIC (0.3 % v/v). THP-1 differentiated macrophages were subjected to phagocytosis assay (**A-C**). Phagocytosis was measured using fluorescein-labeled E. coli (K-12 strain). Data represent mean ± SD (n = 4-5); *p<0.05 compared to vehicle-treated and #p<0.05 compared to enzyme-treated.

PPE significantly potentiated anti-inflammatory cytokine in human cultured macrophages

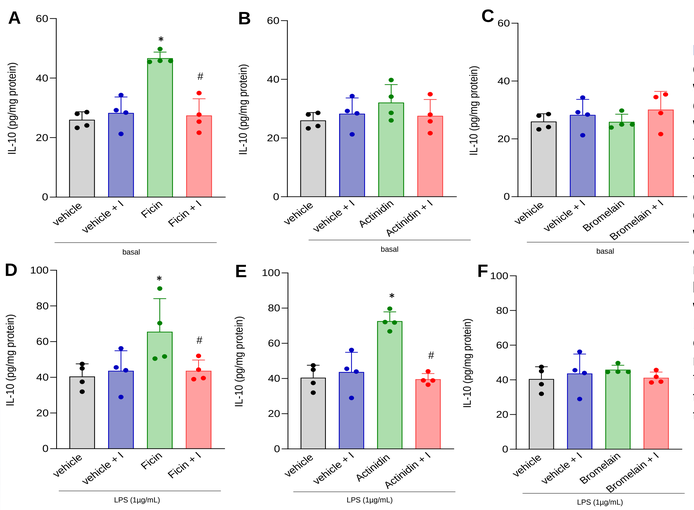

Figure 3. THP-1 cells were differentiated to macrophage with PMA (20 ng/ml, 48 h). The differentiated cells were treated with PPE (vehicle gel 1 % v/v, ficin 10^{-6} % v/v, actinidin 5 X 10^{-4} % v/v and bromelain 10^{-5} % v/v) and PIC (0.3 % v/v). IL-10 cytokine released from THP-1 differentiated macrophages was measured from the cell culture media using ELISA and normalized to total protein of lysate. Cytokine measurements were performed at basal and LPS induced (1µg/mL) conditions (**A-F**). Data represent mean ± SD (n = 4). *p<0.05 compared to vehicle-treated and #p<0.05 compared to enzyme-treated.

PPE significantly potentiated pro-inflammatory cytokine in wound macrophage of early (d3) inflammatory phase

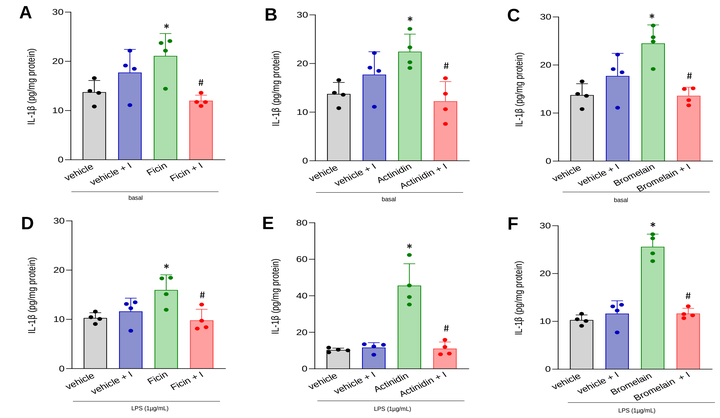

Figure 4. PVA sponges were implanted subcutaneously following incisional wounding in C57BL/6 mice (n = 5-8). Day 3 wound macrophages were harvested from the sponges and treated with PPE (vehicle gel 1 % v/v, ficin 10^{-6} % v/v, actinidin 5 X 10^{-4} % v/v and bromelain 10^{-5} % v/v) and PIC (0.3 % v/v). IL-1β and IL-10 level was measured from the macrophage cell culture media using ELISA and normalized to total protein of lysate. Cytokine measurements were performed at basal and LPS-induced (1µg/mL) conditions (**A-F**). Data represent mean ± SD (n = 4). *p<0.05 compared to vehicle-treated and #p<0.05 compared to enzyme-treated.

PPE attenuated pro-inflammatory and induced anti-inflammatory cytokines in wound macrophage of late (d10) inflammatory phase

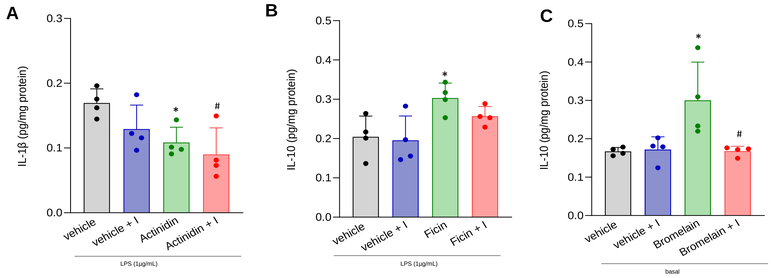

Figure 5. PVA sponges were implanted subcutaneously following incisional wounding in C57BL/6 mice (n = 5-8). Day 10 wound macrophages were harvested from the sponges and treated with PPE (vehicle gel 1 % v/v, ficin 10^{-6} % v/v, actinidin 5 X 10^{-4} % v/v and bromelain 10^{-5} % v/v) and PIC (0.3 % v/v). IL-1β and IL-10 level was measured from the macrophage cell culture media using ELISA and normalized to total protein of lysate. Cytokine measurements were performed at basal and LPS-induced (1µg/mL) conditions (**A-C**). Data represent mean ± SD (n = 4). *p<0.05 compared to vehicle-treated and #p<0.05 compared to enzyme-treated.

SUMMARY OF OBSERVATIONS

1. **PPE induced phagocytosis and efferocytosis in human cultured macrophages**
2. **PPE potentiated pro and anti-inflammatory cytokines at appropriate stages of wound healing**
3. **PPE also attenuated pro and anti-inflammatory cytokines at appropriate stages of wound healing.**

CONCLUSION

This work presents maiden evidence that PPE is capable of modifying wound inflammation by virtue of its protease activity. PPE induces production of anti-inflammatory and pro-angiogenic cytokines and facilitates polarization of wound-site macrophages towards pro-healing phenotype which may help in quality healing.

ACKNOWLEDGEMENTS

- Product Information: PPE represents Ficin, Actinidin and Bromelain products obtained from Swiss American, TX.
- Current studies were supported with funds from Swiss American, TX. Study design and conduct of experiments were not influenced by input from the company.