In vitro anti-inflammatory activities of blacklip abalone (*Haliotis rubra*) in RAW 264.7 macrophages

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**ABSTRACT**

Abalone (*Haliotis sp*) has been used as a traditional functional food for many years by different cultures believing that consumption provides health benefits. We investigated the anti-inflammatory activity from blacklip abalone visceral waste. An extract was prepared from the viscera using digestion with the food grade proteases papain and bromelain followed by anion exchange chromatography (AEC). The anti-inflammatory potential of the extract and AEC fractions were investigated in comparison to a plant-derived positive control (quercetin) through the inhibition of lipopolysaccharide-stimulated nitric oxide production in RAW 264.7 monocytes. Significant anti-inflammatory activity was observed in response to the abalone extract and the unbound AEC material over a concentration (based on collagen) range of 12\textsuperscript{-}96 µg/mL. Overall, results indicated that blacklip abalone extract has anti-inflammatory activity *in vitro*, warranting further investigation into the bioactive constituents that may be potential anti-inflammatory therapeutics.

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1. Introduction

Inflammation is a protective mechanism in response to disease, physical trauma, noxious stimuli by chemical agents, heat, antigen\textendash;antibody reactions and microbial effects (Cheng et al., 2015). Inflammation is a complicated process engaging multifactorial networks of chemical-based signals helping the body to eliminate or limit the spread of injurious agents (Soni et al., 2014). Macrophages play a significant role in inflammation by producing nitric oxide (NO) which mediates many physiological functions, such as nonspecific host defences, anti-microbial defences and anti-tumor activities, as well as pathological processes including organ destruction in some inflammatory and autoimmune diseases (Turini & DuBois, 2002). NO is generated biochemically via oxidation of the terminal guanidine nitrogen of L-arginine by nitric oxide synthase. The inhibition of NO overproduction by blocking inducible nitric oxide synthase...
synthase (iNOS) expression may be a useful strategy in the treatment of various inflammatory disorders (Qian et al., 2012). Overproduction of these factors leads to cell damage and inflammatory disease (Quang et al., 2014); therefore, inhibition of these inflammatory mediators is one of the important strategies in treating inflammatory diseases (Yoon et al., 2012).

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat inflammation and associated pain even though long-term usage of NSAIDs can lead to adverse gastrointestinal outcomes such as peptic ulcers (Takeuchi, 2012). Consequently, there is considerable interest in safer drugs (Prabhu, Nalini, Chidambaranathan, & Kisan, 2011) with alternative medicines from plant and marine sources gaining popularity as therapeutics due to their mild action and fewer side effects (Sun et al., 2016). The discovery of novel bioactive peptides from marine origins with specific cellular targets, may complement the search for promising drug candidates. During the last decade, hundreds of marine-based bioactive molecules have been discovered with several undergoing clinical trials (Mayer, Rodríguez, Taglialetela-Scafati, & Fusetani, 2013; Suleria, Masci, & Globe, 2015). These bioactive molecules show anti-bacterial, anti-fungal, anti-cancer, anti-viral, as well as anti-inflammatory activities that can be used to promote the health status of humankind (Dang, Benkendorff, Green, & Speck, 2015; Sugiura, Nagayama, Kinoshita, Tanaka, & Matsushita, 2015), however, the biological activity of most of the marine compounds is still unclear and under investigation (Blunt et al., 2009).

Blacklip abalone (Haliotis rubra), a single-shelled marine mollusc, is harvested commercially in many international waters and widely cultured in eastern Asia. During the processing of commercial products, the viscera, considered inedible, are discarded and account for approximately 30% of abalone weight. Extracts have been prepared from abalone viscera with anti-inflammatory activity demonstrated in vitro and in vivo (Sun et al., 2010). Anti-inflammatory properties of water extracts and fermented hydrolysate of Haliotis discus hannai Ino have also been reported by Hasnat et al. (2015) using lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. In this study, H. discus hannai Ino water extracts and fermented hydrolysate decreased NO production in LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner at concentrations of 1 to 8 mg/mL protein with no detrimental effect on cell viability. Cheong et al. (2015) also reported the inhibitory effect of H. discus water extracts on LPS-induced NO production in zebrafish by using a fluorescent dye probe. Qian et al. (2012) also reported the anti-inflammatory effects of H. discus hannai intestinal digest in RAW 264.7 macrophage cells. According to their findings, the abalone intestinal digest suppressed LPS-induced production of NO via reduced iNOS expression in a dose-dependent manner.

Drug discovery for the treatment of inflammation often focuses on the decreased production of the pro-inflammatory mediator NO (Qian et al., 2012) as well as iNOS and the regulation of specific cytokines like tumor necrosis factor α (TNF-α) and interleukins 1β (IL-1β) and 6 (IL-6), and transcription factors such as nuclear factor κB (NF-κB) (Sun et al., 2010). In the current research, an extract was prepared from blacklip abalone viscera and fractionated prior to in vitro assessment of anti-inflammatory activity. Cell viability and NO production was measured following treatment of LPS-induced RAW 264.7 macrophages with blacklip visceral extract and fractions.
2. Materials and methods

2.1. Materials

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. The murine macrophage cell line, RAW 264.7 was from the American Type Culture Collection (Rockville, MA, USA). All plasticware for cell culture was either Nunc (In Vitro Technologies, Noble Park North, Australia) or Corning (Sigma-Aldrich). Growth media (RPMI-1640) and fetal bovine serum (FBS) were from Gibco (Invitrogen/Life Technologies Pty Ltd, Mt. Waverley, Australia). Penicillin and Streptomycin (Pen/strep) were from Bio-Whittaker (Edward Keller Australia, Silverwater, Australia). For NO quantification, Griess reagent, LPS from Escherichia coli 0111:B4 and Quercetin were from Sigma-Aldrich. The CellTiter 96® AQeuous Non-Radioactive Cell Proliferation Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt or MTS) was from Promega (Madison, WI, USA). Fresh Australian wild caught blacklip abalone (H. rubra) visceral samples were provided by Tasmanian Seafoods, Hobart, Australia.

2.2. Preparation of extracts

Extracts were prepared using 5.0% w/w food grade proteases papain and bromelain (Enzyme Solutions, Melbourne, VIC, Australia), combined in 1:1 ratio. For the purpose of determining the contribution of the two proteases to the subsequent assay measurements, enzyme-only control digests were prepared using the same concentrations and conditions but with no added abalone. Digests were incubated overnight (14–16 h) at 50°C, inactivated by heating at 95°C for 10 min, cooled on ice and centrifuged (Avanti® J-26XP1; Beckman Coulter, Brea, CA, USA) at 5940 g for 10 min to remove undigested material (pellet). Supernatants were clarified using sequential filtration with 2 µm, 1 µm (Whatman™, GE Healthcare Life Science, Parramatta, NSW, Australia) and 0.45 µm (mixed cellulose ester, Merck Millipore, Bayswater VIC, Australia) filter paper and stored at –20°C. Due to likely interference with bioassays, salt ions were reduced in the abalone hydrolysate and pooled fractions using 3 kDa molecular weight cut-off (MWCO) spin columns (Centrifuge Filter Unit, Merck Millipore, Billerica, MA, USA). Samples (10 mL) were added into the spin column and centrifuged at 3270g for 30 min. This process was repeated using deionized water until salt was at or below 60 mM as determined by conductivity measurements (Metler Toledo-AG conductivity meter, VWR International, Dietikon, Switzerland) using a NaCl standard curve.

2.3.1. Estimation of protein content

Protein content was estimated in all samples and extracts using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as a protein standard, according to manufacturer’s instructions. All assays were performed in triplicate. Absorbance was measured at 562 nm using a Spectra-Max M3 System spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and concentrations determined using the associated instrument software (SoftMax-Pro 6.1).
2.3.2. Estimation of total phenolic contents
The total phenolic content (TPC) in the abalone extract was determined by using the Folin–Ciocalteu method described by Chang et al. (2002). Fifty microliters abalone extract or fractionated samples, sterile water (as the blank) or a standard solution of gallic acid (6.25, 12.5, 25, 50, 100 µg/mL in distilled water) was added to 50 µL 10% Folin–Ciocalteu’s phenol reagent and 50 µL 1 M sodium carbonate solution in triplicate, in a 96-well plate (Nunclon Delta Surface, Thermo Fisher Scientific, Waltham, MA, USA). The plate was incubated for 60 min at room temperature in the dark. The absorbance was measured at 750 nm using a Spectra-Max M3 System spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). TPC in the abalone extract and fractionated samples was expressed as mg gallic acid equivalents per gram abalone extract/fractionated samples.

2.3.3. Estimation of collagen
Total collagen content (based on hydroxyproline detection) was estimated in the extracts using the QuickZyme Biosciences Total Collagen Assay Kit (BioScientific Pty Ltd) according to the manufacturer’s instructions with modifications. Briefly, 125 µL standard (rat tail collagen) or samples were added to 125 µL 12 M HCl and hydrolyzed for 20 h at 95°C in screw capped tubes. Following the hydrolysis, 35 µL hydrolyzed samples and standards were transferred to a 96-well plate provided for the assay, mixed with 75 µL assay buffer and incubated at room temperature (∼21°C) for 20 min. Seventy-five microliters of detection reagent was then added to each well and the resulting solution mixed and incubated at 60°C for 30 min. Absorbance was read at 570 nm and a linear standard curve constructed to interpolate the total collagen concentration. All assays were performed in duplicate.

2.4. Separation of blacklip abalone extracts using anion exchange chromatography via fast protein liquid chromatography
Q Sepharose™ Big Beads (372.5 mL) was packed into an empty GE-XK 26/70 column (700 mm × 26 mm, GE Healthcare Life Science, Chicago, IL, USA). The packed column was attached to a fast protein liquid chromatography (FPLC) system (ÄKTA Lab-Scale Systems, GE Healthcare Life Sciences, Chicago, IL, USA). The column was equilibrated with deionized water (Buffer A) before a sample of approximately 800–900 mg GAG (sulphated polysaccharide basis) was loaded onto the column. Flow rate was set at 5 mL/min with a column pressure of 1 MPa. After isocratic washes of buffer A over 660 minutes (3300 mL), 15 and 50 mL fractions were collected on commencement of a 0–2 M NaCl linear gradient delivered over 330 min (1650 mL). Elution was monitored at 280 nm for protein content (Talaei et al., 2016), and 206 nm for glycosaminoglycan detection (Marks et al., 2001). Conductivity was also monitored throughout the run. The collected fractions were pooled on the basis of their interactions with dimethylmethylene blue (DMMB) dye. Overall, five anion exchange chromatography (AEC) pools were prepared from the linear NaCl gradient (numbered 1–5) along with the unbound material, initial column wash and final column wash. For further analysis, all pooled samples were desalted using
3 kDa MWCO spin columns and washed using deionized water to reduce salt concentration to 60 mM or less.

2.5. **Cell culture studies**

2.5.1. **Culture of RAW 264.7 cells**

RAW 264.7 cells were cultured in growth media comprised of RPMI-1640 supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% FBS. Cells were grown in a humidified atmosphere with 5% CO₂ at 37°C. A mycoplasma test was performed routinely and there was no bacterial contamination.

2.5.2. **NO assay**

NO production, measured as nitrite, was determined using the Griess Reagent. RAW 264.7 cells were seeded into 96-well plates at a density of 50,000 cells/well (in 100 µL) along with 20 µL 300 ng/mL LPS or 20 µL Quercetin (0.3–100 µM), with or without abalone extract or abalone AEC pools (in triplicate applications of 12, 24, 48, 96, 192 and 375 µg/mL on a collagen basis). The cells and various treatments were incubated at 37°C and 5% CO₂ for 48 h.

After 48 h, 50 µL cell media was removed from each well and transferred into a new 96-well plate. Fifty microliters of various concentrations of sodium nitrite standard were also added in either duplicate or triplicate to the 96-well plate in preparation for the standard curve. Finally, 50 µL of Griess Reagent were added to each well for 15 min and absorbance measured at 540 nm. The nitrite concentration of each cell treatment was extrapolated from the sodium nitrite standard curve.

2.5.3. **Cell viability**

Cell viability was measured using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay for determining the number of viable cells. Following cell treatments in 96-well plates, 20 µL of MTS solution were added to 140 µL of media and incubated with the cells for 120 min at 37°C. Absorbance was measured using a Spectra-Max M3 System spectrophotometer at 492 nm.

2.6. **Statistical analyses**

All statistical analyses were conducted using a one-way analysis of variance with Dunnett’s comparison tests or unpaired t-tests. These calculations were carried out using GraphPad Prism 5 Software for Windows (GraphPad Software, San Diego CA, www.graphpad.com). Significance was observed at *p* < .05.

3. **Results and discussion**

3.1. **Preparation of abalone extract and fractionation via AEC**

Marine processing waste, often discarded due to limited know-how, market constraints and technological barriers, is a good source of bioactive molecules (Hayes & Tiwari, 2015). One of the biggest challenges and most important research areas in this field is the isolation and purification of these bioactive molecules for the development of
Figure 1. Total protein, collagen and phenolic content in abalone extracts and AEC pools. (A) Total protein; (B) total collagen and (C) TPC in extract (Ex), unbound material (Un), initial column wash (IW), AEC pools 1–5 (P1–P5) and final column wash (FW) expressed as mean ± standard deviation.
Figure 2. Screening of cell viability in RAW 264.7 macrophages treated with LPS and abalone extract or anion exchanged (AEC) pools. RAW 264.7 cell viability, following 48-h treatment with 300 ng/mL LPS and 375, 196, 9, 48, 24 and 12 µg/mL abalone extract or AEC pools, was measured in triplicate (n = 3) using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay. Cell viability is expressed as the mean percentage viable cells (± standard deviation) compared to LPS control. Statistical significance determined using a one-way analysis of variance with Dunnett’s Multiple Comparison Test compared to LPS control with *p < 0.05.
Figure 3. Screening of anti-inflammatory activity in RAW 264.7 cells by abalone extract and AEC pools. Anti-inflammatory activity in RAW 264.7 cells, following 48-h treatment with 300 ng/mL LPS and 375, 196, 9, 48, 24 and 12 µg/mL abalone extract or AEC pools, was measured in triplicate ($n = 3$) and expressed as mean percentage decrease in nitric oxide production (assayed as nitrite) ± standard deviation compared to LPS treatment control.
various therapeutic products (Orgueira et al., 2003). In the current research, an extract was prepared from blacklip abalone viscera using papain and bromelain enzymes and subjected to anion exchange chromatography. Total protein, collagen and phenolic contents were estimated in the extract and all AEC pools. In Figure 1, total protein, collagen and phenolic contents were higher in the extract and AEC-unbound material as compared to other AEC pools. Therefore, it appears as though the majority of peptides in the extract are cations, carry no charge or are weak anions.

3.2. Effect of abalone extract and AEC pools on inflammation and cell viability in vitro

When cells are activated with pathogenic substances, macrophages initiate and regulate inflammatory responses through a broad range of inflammatory mediators. LPS are among the most effective macrophage activators, and it has been known that LPS-stimulated macrophages produce excessive inflammatory mediators that have been in close relation with elevated anti-inflammatory response (Meng and Lowell, 1997). Therefore, the anti-inflammatory effect of the abalone extract and the AEC pools was investigated using LPS-stimulated RAW 264.7 cells. Inflammation was indicated by the cellular production of NO as measured using the Griess assay. A decrease in the production of NO by the positive control (Quercetin) or by cell treatment with abalone extracts or AEC pools, indicated anti-inflammatory activity. To ensure that the decrease in NO production was not associated with cell toxicity or cell death, RAW 264.7 cell viability was measured in response to all treatments using a screening MTS assay.

In Figure 2, cell viability results show that the extract and some AEC pools displayed some cytotoxic effects over the treatment concentration range 12–375 µg/mL (on a
Figure 5. Anti-inflammatory activity by abalone extract and AEC pools in RAW 264.7 macrophages. Anti-inflammatory activity in RAW 264.7 cells, following 48-h treatment with 300 ng/mL LPS and 375, 196, 9, 48, 24 and 12 µg/mL abalone extract or AEC-unbound material, was measured in three independent experiments in triplicate ($n = 9$) and expressed as mean percentage decrease in NO production (assayed as nitrite) ± standard deviation compared to LPS treatment control. Statistical significance determined for extract and unbound material using unpaired t-tests with 100 µM quercetin (*$p < 0.05$) and 30 µM quercetin (#$p < 0.05$).
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collagen basis), when compared to LPS control cell viability. Cell viability decreased the most (over 90%) in response to 375 µg/mL AEC pool 5. The extract also decreased cell viability by approximately 35 and 30% in response to 375 and 195 µg/mL treatments; cell viability also decreased 10–20% in response to AEC pools 1 (12 and 24 µg/mL) and 2 (12–96 µg/mL). In Figure 3, the extract and AEC-unbound material suppressed LPS-induced production of NO in a dose-dependent manner. Anti-inflammatory activity without cell toxicity was observed in response to 12–96 µg/mL abalone extract and 96–375 µg/mL AEC-Unbound material.

To further investigate the anti-inflammatory effect of the extract and AEC-unbound material and related cytotoxicity, three additional in vitro experiments were performed in comparison to quercetin. Figure 4 shows the mostly cytotoxic effect of the extract (at 375, 96, 24 and 12 µg/mL) on cell viability whilst the AEC-unbound material (at 195, 96, 48 and 24 µg/mL) and quercetin (at 100, 30, 10, 3 and 1 µM) significantly increased or did not change the percentage of viable cells compared to the LPS control. The anti-inflammatory effects of these treatments are presented in Figure 5 and show that 100 and 30 µM quercetin and 375, 195 and 96 µg/mL abalone extract decreased nitric oxide production in a similar manner. However, in the absence of decreased cell viability, the AEC-unbound produced an anti-inflammatory response similar to 30 µM quercetin offering the most promising source of anti-inflammatory abalone molecules without cytotoxicity.

A previous study has also confirmed that abalone extracts have nitrite radical scavenging activities. In a study by Hasnat et al. (2015), an abalone extract showed dose-dependent nitrite scavenging activities ranging from 25.14–36.28% at concentrations of 1–8 mg/mL w/v computing with α-tocopherol of 100 µg/mL. In a study by Rho et al. (2015), involving Fourier transform infrared spectroscopy (FTIR) analysis of abalone extract, glycoprotein and polysaccharide-rich fractions were consistent with anti-inflammatory and anti-oxidant bioactivities. Furthermore, abalone have been found to contain high contents of polyphenols and gamma-aminobutyric acid that is dependent upon how much seaweed abalone consume (Dang, Li, Speck, & Benkendorff, 2011; Stott, Takeuchi, Koike, Imada, 2003).

4. Conclusion

To the best of our knowledge, there is no published research addressing the anti-inflammatory effect of blacklip abalone viscera. In this in vitro study, NO production was inhibited by molecules separated from blacklip abalone visceral extract, in particular the AEC-unbound material at a concentration range of 48–375 µg/mL (as measured on a collagen basis). Further strategies must be considered to better separate blacklip abalone visceral extract and improve the cellular responses to anti-inflammatory molecules present. Further research is also needed to investigate other anti-inflammatory markers, like cytokine secretion, that will help to elucidate the mechanism.

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experiments and analysis. Hafiz Ansar Rasul Suleria and Simone A. Osborne wrote the manuscript. Glenda Gobe supervised and provided advice to Hafiz Ansar Rasul Suleria, contributed reagents and edited the manuscript.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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