

Anti-cyclooxygenase effects of lipid extracts from the New Zealand green-lipped mussel, *Perna canaliculus*

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Abstract

Total lipid extracts of *P. canaliculus* (a bivalve marine mollusc native to New Zealand, commonly called the green-lipped mussel) and *Mytilus edulis* (commonly called the common blue mussel) moderately inhibited ovine COX-1 and COX-2 pure enzymes *in vitro*. The inhibition was increased after the mussel extracts were saponified by KOH hydrolysis. Protease- and protease–lipase-hydrolysed lipid extracts of *P. canaliculus* exhibited similarly strong COX inhibition as the KOH-hydrolysed extract. Lyprinol® (a commercial extract from *P. canaliculus*) also exhibited strong inhibition of both COX isoforms, an effect that was increased 10-fold upon subsequent hydrolysis. In contrast, fish oil was not as anti-COX active as Lyprinol. The Lyprinol free fatty acid fraction, and to a lesser extent the Lyprinol triglyceride fraction, were the only lipid classes of Lyprinol to exhibit strong inhibition of the COX isoforms. The purified PUFA extracts were all bioactive, potently inhibiting COX-1 and COX-2. Incubation of Lyprinol in the absence of exogenous arachidonic acid (AA) showed the appearance of alternate prostaglandin metabolites, confirming Lyprinol PUFA as a competitive substrate inhibitor of AA metabolism.

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1. Introduction

Cyclooxygenase (COX) are lipid metabolising enzymes that catalyse the oxygenation of polyunsaturated fatty acids (PUFA), preferably arachidonic acid (AA), to form the prostanoids, which are potent cell-signalling molecules associated with the initiation, maintenance and resolution of inflammatory processes (Charlier and Michaux, 2003). Selective modulation of the many prostanoids has important therapeutic potential for the treatment of inflammation and inflammatory conditions such as rheumatoid arthritis. Traditional drug treatments for relieving the pain and swelling of inflammation include aspirin, indomethacin and other non-steroidal anti-inflammatory drugs (NSAIDs). Unfortunately, NSAIDs are also noted for undesir-

able gastrointestinal side effects related to their use (Parente and Perretti, 2003). For this reason, selective COX-2 inhibitors (the ‘Coxibs’) have come to attention in recent years. As coxibs are increasingly used in clinical practice, it appears that they too exhibit side effects, most notably in relation to cardiovascular disturbances. As a result, medical researchers are looking for safer, more efficacious alternatives to both the traditional NSAIDs and the more recent COX-2 selective inhibitors.

One natural alternative for the treatment of inflammation is the New Zealand green-lipped mussel, *Perna canaliculus* (Bivalvia: Mytilidae). This marine mollusc has demonstrated gastroprotective, antihistaminic, antioxidant, anticytokine, anti-inflammatory and antiarthritic properties (Gibson et al., 1980; Rainsford and Whitehouse, 1980; Kosuge et al., 1986; Whitehouse et al., 1997; Mani et al., 1998; Tan and Berridge, 2000); with bioactive principles reportedly residing in the protein, polysaccharide and lipid fractions of the mussel (Miller and

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Ormrod, 1980; Couch et al., 1982; Miller et al., 1993; Mani et al., 1998; Tan and Berridge, 2000). Commercial preparations of *P. canaliculus* include the stabilised freeze-dried anti-inflammatory mussel powder, Seatone® (Kosuge and Sugiyama, 1989), and Lyprinol® which is the mussel “oil” obtained by supercritical fluid extraction of the stabilised mussel powder using liquefied carbon dioxide (Macrides and Kalafatis, 2000). The lipid-rich Lyprinol extract shows significant anti-inflammatory activity when given both therapeutically and prophylactically *po* to Wistar and Dark Agouti rats developing either antigen-induced polyarthritis or collagen (II)-induced auto-allergic arthritis, with ED₅₀ values ≤ 15 mg/kg, compared with naproxen ED₅₀ ≥ 25 mg/kg or with other therapeutic oils (flaxseed, evening primrose, fish) ED₅₀ ≥ 1800 mg/kg (Whitehouse et al., 1997). Lyprinol exhibits little or no efficacy in acute irritation assays (carrageenin, kaolin, histamine); but in contrast to classical NSAIDs, Lyprinol is non-gastrotoxic in diseased-stressed rats at 300 mg/kg *po*, and has no effect on *ex vivo* platelet aggregation triggered by arachidonate or ADP, either in rats (50 mg/kg) or humans (150 mg/kg) (Whitehouse et al., 1997). *In vitro* biochemical testing of Lyprinol has been undertaken to more fully understand the anti-inflammatory mechanism of the lipid extract, and much of this work has focused on the eicosanoid biochemical pathways found in inflammatory cells. Inhibition of leukotriene-B₄ production by Lyprinol has been observed in calcium and ionophore-stimulated human neutrophils (Whitehouse et al., 1997) and also in interleukin-4-induced human monocytes (Dugas, 2000). Inhibition of prostaglandin-E₂ production in activated human macrophages has also been observed (Whitehouse et al., 1997). Lyprinol, therefore, appears to exert some effect on the arachidonic acid cascade.

In this study, the anti-inflammatory properties of various lipid extracts of the New Zealand green-lipped mussel *P. canaliculus* was studied *in vitro* in relation to arachidonic acid metabolism by COX enzymes. Lyprinol and its component ω3 PUFAs were also tested for anti-COX activity, and this activity was compared to the common blue mussel *Mytilus edulis*, to fish oil, and to the known NSAID indomethacin. The production of COX metabolites from Lyprinol was also investigated by gas chromatography mass spectroscopy (GCMS).

2. Materials and methods

2.1. Chemicals

Pure standards of AA, various PUFA, prostaglandins and purified COX enzymes were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Indomethacin, 2,7-dichlorofluorescein, 2,7-dichlorofluorescein diacetate, hematin and phenol were obtained from Sigma Chemical Company (St Louis, MO, USA). Lipid standard 18-5A was obtained from NuCheck (Minneapolis, MI, USA). Neutrase® 0.8L (endoprotease) and Novozym® 871L (*sn*-1, *sn*-3-specific lipase) were donated by Novo Nordisk (Bagsvaerd, Denmark). Silica gel for column chromatography (Kieselgel 60, 230–400 mesh), thin

layer chromatography (TLC) plates (Kieselgel 60 G, 5–40 gm), and pre-prepared TLC plates (Kieselgel 60 F254 nano DC) were sourced from Merck (Darmstadt, Germany). Silica Sep-Pak cartridges and 0.45 μm polytetrafluoroethylene (PTFE) syringe filters were obtained from Waters Corporation (Milford, MA, USA). High performance liquid chromatography (HPLC)-grade methanol and other solvents used for lipid extraction and analysis were supplied by Selby Biolab (Clayton, Victoria, Australia). All other general laboratory chemicals and reagents were of analytical quality and obtained through Merck (Darmstadt, Germany).

2.2. Lipid test samples

P. canaliculus and *M. edulis* mussel samples were collected from Hallam Cove, New Zealand, March 2000, by Dr Henry Kaspar (Cawthron Institute, New Zealand). The whole body mussel meat was refrigerated (4 °C) then processed by freeze-drying within 24 h of collection. Lyprinol® was supplied by McFarlane Laboratories (Melbourne, Victoria, Australia). Blackmores Fish Oil 1000® was purchased from a Melbourne pharmacy. Lyprinol, fish oil, and a variety of purified PUFA were dissolved in HPLC-grade methanol ready for biochemical analysis.

2.3. Lipid extraction

Total lipid extracts of the freeze-dried mussel meat of *P. canaliculus* and *M. edulis* were obtained using a standard chloroform–methanol extraction procedure (Bligh and Dyer, 1959). Three grams of freeze-dried meat was used in each respective extraction (from approximately 20 combined individuals). The extracted lipid material was dissolved in HPLC-grade methanol ready for biochemical analysis, or taken for further processing (saponification).

2.4. Hydrolysis of total lipid extracts

P. canaliculus and *M. edulis* total lipid extracts were saponified by KOH hydrolysis according to the method of Christie (1989). Pyrogallol (5%) was included in the hydrolysis mixture as an antioxidant. Saponified extracts containing the total free fatty acid fraction were dissolved in HPLC-grade methanol ready for biochemical analysis.

2.5. Enzyme assisted extractions of *P. canaliculus*

The protease *P. canaliculus* lipid extract (P) was prepared by addition of Neutrase® 0.8 L (5 mL) to freshly thawed whole mussel in shell (1.5 kg, purchased from a local Melbourne fishmonger) with incubation (2 h, 40 °C). The protease–lipase *P. canaliculus* lipid extract (PL) was prepared by digestion with Neutrase, followed by cleavage of esterified fatty acids with Novozym 871 L® (0.3 mL of lipase added to 620 mL of liquid mixture previously treated with neutrase) with incubation (3 h, 23 °C). Lipids were isolated from the respective aqueous digestions by freeze-drying followed by solvent extraction

using diethyl ether. Silica gel TLC analysis was performed on the extracts utilising a solvent system of hexane–ether–acetic acid (70:30:2 v/v/v) and visualised by spraying with phosphomolybdic acid before charring at 100 °C for 5 min. The lipid extracts were evaporated to dryness and each re-dissolved in HPLC-grade methanol ready for biochemical analysis.

2.6. Hydrolysis of Lyprinol

Lyprinol was saponified by KOH hydrolysis according to the method of Christie (1989). Pyrogallol (5%) was included in the hydrolysis mixture as an antioxidant. Saponified Lyprinol containing the total free fatty acid fraction was dissolved in HPLC-grade methanol ready for biochemical analysis.

2.7. Preparation of Lyprinol lipid classes

Separation of Lyprinol (1.07 g) into its constituent lipid classes was achieved using open column chromatography on a stationary phase of silica gel, and a polarity gradient of methyl-*tert*-butyl ether (MTBE) in hexane. Column fractions of sterol esters, triglycerides, free fatty acids, sterols and phospholipids were combined to give one fraction for each lipid class. Each lipid class was evaporated to dryness and weighed to obtain percentage composition. The purity of the separated lipid classes was determined by silica gel TLC utilising a solvent system of hexane-MTBE-acetic acid (80:20:2 v/v/v), and visualised by spraying with phosphomolybdic acid before charring at 100 °C for 5 min. The separated Lyprinol lipid classes were diluted in HPLC-grade methanol ready for biochemical analysis, or taken for further processing (methylation).

2.8. Methylation of Lyprinol lipid classes

The individual lipid classes were hydrolysed with 3.8% KOH and converted to fatty acid methyl ester (FAME) by the addition of boron trifluoride. Separation of the FAME by double bond number was achieved using silver impregnated silica TLC plates and a solvent system of hexane-MTBE (40:60 v/v), and visualised at 254 nm after spraying with 2,7-dichlorofluorescein. The individual FAME bands were extracted from the silica twice with MTBE-hexane (1:1 v/v), filtered through a 45 µm PTFE filter, and evaporated to dryness.

Extracted residues were treated with an ethereal solution of diazomethane containing 10% by volume of methanol. The treated samples were evaporated to dryness under nitrogen at room temperature. Residues were reconstituted in ethyl acetate (200 µL) and the mixture was heated in a sealed tube at 80 °C for 20 min before being transferred to an autosampler vial prior to GCMS analysis. A second procedure for methylation involved treatment with *N*-trimethylsilyl-trifluoroacetamide (MSTFA, 35 µL, Pierce, Rockford, IL, USA) and toluene (65 µL).

2.9. GCMS analysis

Electron impact (EI)-GCMS was performed on a Hewlett Packard 6890 GC-5973 MSD with a 7683 injector (Palo Alto,

USA). The GC was equipped with a BPX5 column (12 m×0.2 mm, 0.25 µm film thickness, SGE, Melbourne, Australia) and used helium as the carrier gas with a constant flow of 1 mL/min. The oven temperature was held at 75 °C for 2 min then increased at 30 °C/min to 300 °C with a final holding time of 9 min. Injections of 2 µL were pulsed splitless with a nominal head pressure of 60 kPa pulsed to 170 kPa for 1 min after injection. A scan range of 50–650 Da at 1.0 scan/s was used.

Fatty acids, sterols and their metabolites were identified by comparison of their EI mass spectra with those of authentic standards, where available. In the absence of suitable standards of the proposed metabolites, assignments were supported by response to methylating reagents, retention time and mass spectral data. Assignments were based on EI mass spectral fragmentation, on positive-ion chemical ionisation assigned molecular ions, and on common fragmentation pathways established for structurally analogous compounds.

2.10. Cyclooxygenase inhibition assay

The cyclooxygenase inhibition assay was performed according to a modified method of Larsen et al. (1996). The oxidation of leuco-dichlorofluorescein (1-DCF) in the presence of phenol by the hydroperoxide formed in the cyclooxygenase reaction can be used as a sensitive spectrophotometric assay for PGH-synthase activity (Larsen et al., 1996). Leuco-2,7-dichlorofluorescein diacetate (5 mg) was hydrolysed at room temperature in 1 M NaOH (50 µL) for 10 min, then 1 M HCl (30 µL) was added to neutralise excess NaOH before the resulting 1-DCF was diluted in 0.1 M Tris-buffer, pH 8. Cyclooxygenase enzyme (COX-1 or COX-2) was diluted in 0.1 M Tris-buffer, pH 8, so that a known aliquot gave an absorbance change of 0.05/min in the test reaction. Test samples (or the equivalent volume of methanol, 20 µL) were pre-incubated with enzyme at room temperature for 5 min in the presence of hematin. Pre-mixed phenol, 1-DCF and arachidonic acid were added to the enzyme mixture to begin the reaction, and to give a final reaction mixture of arachidonic acid (50 µM), phenol (500 µM), 1-DCF (20 µM) and hematin (1 µM) in 1 mL final volume of 0.1 M Tris-buffer, pH 8. The reaction was recorded spectrophotometrically over 1 min at 502 nm. A blank reaction mixture was analysed in the spectrophotometer reference cell against each test reaction to account for any non-enzymatic activity attributed to the test sample. This blank consisted of the reaction mixture without the addition of enzyme. Lyprinol was investigated at multiple concentrations and exhibited 50% inhibition of the enzyme reaction at approximately 1 µg/mL (final concentration), therefore the other lipid samples were tested at this final concentration for comparison.

2.11. Cyclooxygenase metabolite analysis by GCMS

Cyclooxygenase enzyme (COX-1 or COX-2), phenol and hematin in 0.1 M Tris-buffer, pH 8 were pre-incubated at 37 °C for 2 min before addition of arachidonic acid [or alternatively Lyprinol] diluted in 0.1 M Tris-buffer, pH 8, to give a final

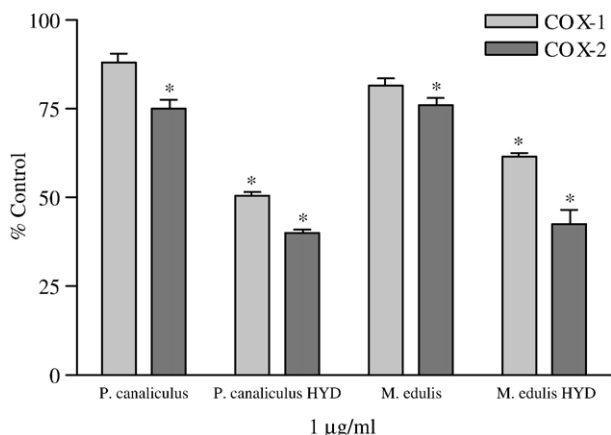


Fig. 1. Inhibition of COX-1 and COX-2 by *P. canaliculus* and *M. edulis* total lipid extracts at 1 µg/mL (mean±SEM, $n=3$). *Significantly different from methanol control based on one way ANOVA with Dunnett's post test ($p<0.05$). Hydrolysed lipid extract (HYD).

reaction mixture of phenol (500 µM), hematin (1 µM), arachidonic acid (80 µM) [or the equivalent weight of Lyprinol (24.36 µg/mL solution)], and 35 units of purified cyclooxygenase enzyme (COX-1 or COX-2). The reaction was run for 15 min and terminated with citric acid (0.23 M) to pH 3.5. The metabolites were then extracted with 5 mL of HPLC-grade chloroform. Each reaction was performed in triplicate and the extracted mixtures combined before separation by TLC to allow ample metabolite quantity for GCMS analysis.

Each extracted mixture was evaporated with nitrogen to approximately 200 µL and applied as a single band to a 10×20 cm silica gel (Kiesel gel 60) TLC plate. The metabolites were separated on a mobile phase of chloroform–methanol–acetic acid–water (90:8:1:0.8 v/v/v/v) and visualised by UV lamp at 254 nm, and the plate divided into five individual bands. Each band was scraped from the plate, extracted with 5 mL of chloroform-methanol (2:1 v/v) and centrifuged for 10 min to sediment the silica particles. Each fraction was filtered from the silica using a PTFE filter and washed through with 2 mL of chloroform before being evaporated under nitrogen. Extracted fractions were treated with an ethereal solution of diazomethane containing 10% by volume of methanol. The treated fractions were evaporated to dryness under nitrogen at room temperature. Fractions were reconstituted in ethyl acetate (200 µL) and the mixture was heated in a sealed tube at 80 °C for 20 min before being transferred to an autosampler vial prior to GCMS analysis. Electron impact (EI)-GCMS was performed as described above.

2.12. Statistical analysis

For the enzyme inhibition assay, the absorbance per min for each test was calculated over the first 60 s of the reaction. Each reaction was performed in triplicate and the results expressed as the mean±SEM ($n=3$) percentage of control, or percentage inhibition of control. Statistical significance between multiple sample groups was analysed by one-way ANOVA, with Dunnett's post hoc test when results were significant

($p<0.05$). Outlier results in the data were detected and removed using Grubbs outlier test.

3. Results

3.1. Inhibition of COX by *P. canaliculus* and *M. edulis* lipid extracts

Cyclooxygenase-1 and COX-2 were inhibited moderately by both *P. canaliculus* (COX-1, 12%; COX-2, 25%) and *M. edulis* (COX-1, 18%; COX-2, 24%) total lipid extracts at 1 µg/mL (Fig. 1). When these extracts were hydrolysed to their total free fatty acid fractions, significant inhibition of both COX isoforms was observed for *P. canaliculus* (COX-1, 49%; COX-2, 60%) and *M. edulis* (COX-1, 38%; COX-2, 57 %).

3.2. Inhibition of COX by *P. canaliculus* (P) and (PL) lipid extracts

At 1 µg/mL, the protease (COX-1, 57%; COX-2, 47%) and protease–lipase (COX-1, 67%; COX-2, 62%)-treated *P. canaliculus* exhibited strong, non-selective inhibition of both COX isoforms (Fig. 2). The (P) extract displayed significantly less inhibition overall than the (PL) extract ($p<0.05$). Analysis by TLC of the enzyme-treated extracts shows a decrease in triglycerides with concomitant increase in free fatty acids after combined protease–lipase treatment.

3.3. Inhibition of COX by Lyprinol and Lyprinol lipid classes

The CO₂ supercritical fluid lipid extract of *P. canaliculus*, Lyprinol, exhibited strong, concentration dependent inhibition of both COX-1 and COX-2, and was more inhibitory than the traditional NSAID COX inhibitor, indomethacin, at comparable concentrations (Fig. 3). Upon examination of the IC₅₀ inhibitory concentration values (Fig. 4), hydrolysed Lyprinol exhibited approximately 10 times more inhibitory activity against COX than non-hydrolysed Lyprinol. Lyprinol (COX-1, 61%; COX-2,

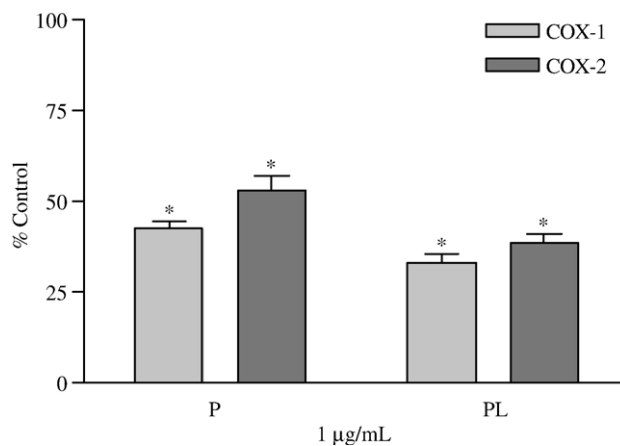


Fig. 2. Inhibition of COX-1 and COX-2 by enzyme-treated *Perna canaliculus* (mean±SEM, $n=3$). *Significantly different from methanol control based on one way ANOVA with Dunnett's post test ($p<0.05$). Protease extract (P); protease–lipase extract (PL).

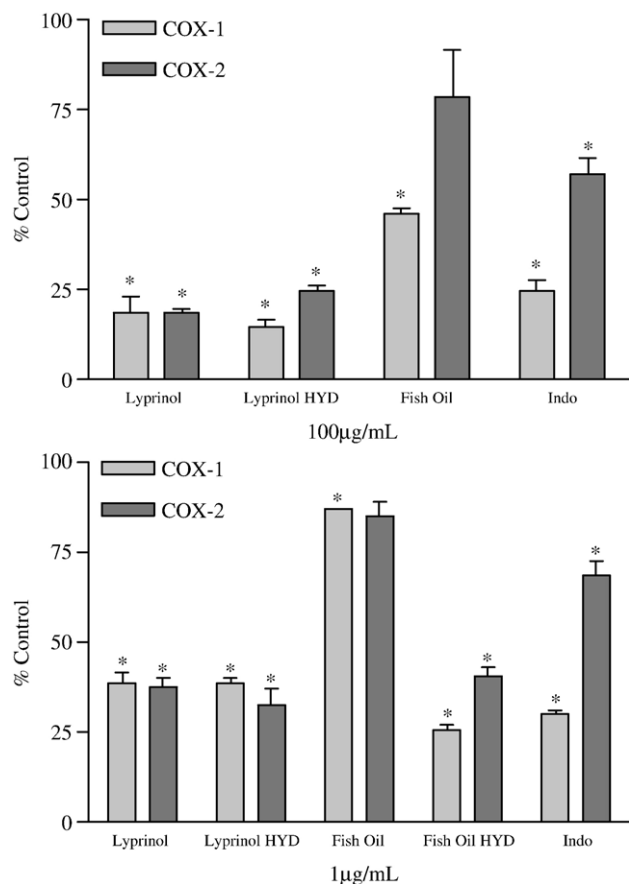


Fig. 3. Inhibition of COX-1 and COX-2 by Lyprinol, fish oil, and indomethacin at 100 and 1 µg/mL (mean ± SEM, $n=3$). *Significantly different from methanol control based on one way ANOVA with Dunnett's post test ($p<0.05$). Hydrolysed (HYD), indomethacin (Indo).

62%) was also more effective at inhibiting COX than fish oil (COX-1, 13%; COX-2, 15%), although once fish oil was hydrolysed (COX-1, 74%; COX-2, 59%) it exhibited similar activity to that of Lyprinol and hydrolysed Lyprinol at 1 µg/mL.

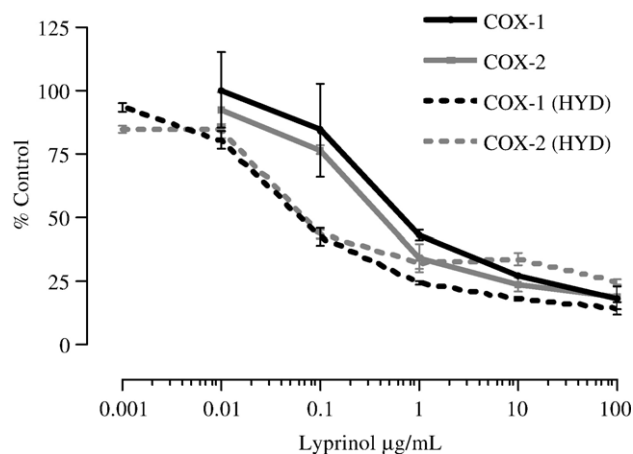


Fig. 4. Inhibition of COX-1 and COX-2 by different concentrations of unhydrolysed and hydrolysed Lyprinol (mean ± SEM, $n=3$). IC_{50} values for Lyprinol were 0.7 µg/mL for COX-1 and 0.45 µg/mL for COX-2. IC_{50} values for hydrolysed (HYD) Lyprinol were 0.07 µg/mL for COX-1 and COX-2, respectively.

Table 1
Lipid class composition of Lyprinol

Lyprinol lipid class	Solvent elution (% MTBE in hexane)	Total Lyprinol (%)
Sterol esters	1–5	5.8
Triglycerides	5–10	26.4
Free fatty acids	10–25	53.7
Sterols	25–50	8.0
Phospholipids (polar lipids)	50–100*	6.1

*Includes 100% methanol fraction (to remove all remaining polar material from the column).

Free fatty acids comprised 54% of the Lyprinol fraction, with triglycerides the next most abundant lipid class comprising 26% (Table 1). Phospholipids, sterol and sterol esters comprised approximately equal amounts of the remaining lipid fraction. The free fatty acid fraction from Lyprinol exhibited the highest inhibition of all the Lyprinol lipid classes, and inhibition was high for both COX-1 (78%) and COX-2 (70%) (Fig. 5). The triglyceride fraction exhibited approximately 50% inhibition of both isoforms (COX-1, 43%; COX-2, 52%). There was limited inhibition of COX by the other Lyprinol lipid classes ($\leq 32\%$). In particular, there was no significant inhibition of COX-1 by the sterol ester, sterol and phospholipids fractions, but there was moderate inhibition of COX-2 by these lipid classes (22%, 23% and 32%, respectively).

The free fatty acid and triglyceride fractions of Lyprinol were found to be abundant in monounsaturated and polyunsaturated fatty acids (Table 2). Saturated fatty acids accounted for over 45% of the total lipid composition of both fractions. The commercially sourced eicosapentaenoic acid (EPA, COX-1, 92%; COX-2, 91%), stearidonic acid (SA, COX-1, 94%; COX-2, 85%), $\omega 3$ -arachidonic acid ($\omega 3$ -AA, COX-1, 95%; COX-2, 89%), $\omega 6$ -arachidonic acid ($\omega 6$ -AA, COX-1, 97%; COX-2, 85%) and α -linolenic acid (α -LNA, COX-1, 92%; COX-2, 85%) all exhibited strong inhibition of COX-1 and COX-2 (Fig.

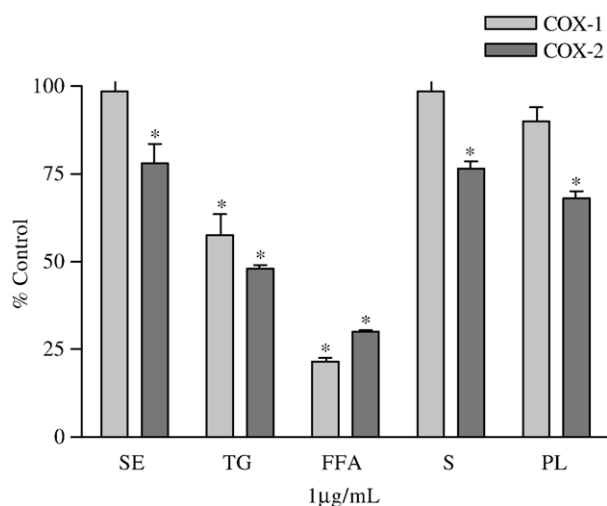


Fig. 5. Inhibition of COX-1 and COX-2 by Lyprinol lipid classes (mean ± SEM, $n=3$). *Significantly different from methanol control based on one way ANOVA with Dunnett's post test ($p<0.05$). Sterol esters (SE); triglycerides (TG); free fatty acids (FFA); sterols (S); phospholipids (PL).

6). Adrenic acid (AdA, COX-1, 76%; COX-2, 75%) was slightly less inhibitory of the COX isozymes than the previously tested fatty acids. Docosahexaenoic acid (DHA, COX-1, 70%; COX-2, 85%) and docosapentaenoic acid (DPA, COX-1, 65%; COX-2, 95%), and especially eicosatrienoic acid (ETA, COX-1, 30%; COX-2, 77%) and docosatrenoic acid (DTA, COX-1, 51%; COX-2, 84%), exhibited less inhibition of COX-1, although inhibition of COX-2 was strong.

3.4. Cyclooxygenase metabolite analysis by GCMS

Incubation of ω 6-AA with COX-1 resulted in production of prostaglandin products as indicated by GCMS analysis (Fig. 7). In relation to the fractions scraped from the TLC plate, fractions 3 and 4 contained multiple prostaglandin peaks, but because of the sample complexity these have not been identified

Table 2
Fatty acid composition of the FFA and TG fractions from Lyprinol

Fatty acid	Percentage FAME in total fraction	
	FFA fraction	TG fraction
4,8,12-trimethyl 10:0		0.004
14:0	8.22	3.61
15:0	1.01	0.78
16:0	38.28	31.18
16:0/17:0		1.25
14-methyl 16:0		0.004
17:0		2.09
18:0	4.8	7.95
20:0	0.07	
20:0/20:1 ω -9*		12.59
16:1 ω -7		0.09
18:1	9.13	12.34
18:1 ω -9/18:2*		0.08
20:1 (2 isomers)	4.75	
20:1 ω -9/20:2 ω -7*		0.05
16:2 ω -4	1.82	0.02
16:2 ω -6	0.56	0.14
17:2		0.005
18:2	7.64	0.33
20:2	0.52	
20:2 ω -9		1.16
20:2/20:3*	1.62	
21:2		0.002
22:2	0.13	1.81
22:2/22:3*	0.38	
16:3	0.15	0.78
18:3 ω -3	5.5	1.67
18:3 ω -6	0.06	0.32
18:3 ω -3/18:4 ω -3*	1.83	
20:3	4.13	0.80
22:3	1.64	0.55
18:4 ω -3	0.04	1.27
20:4 ω -3		0.26
20:4 ω -6		0.56
22:4/22:5*		0.02
20:5 ω -3	1.14	1.83
21:5	0.05	0.06
22:5	0.03	
22:6 ω -3	0.38	0.05
22' fatty acids		0.24
TOTAL	94.15	83.90

*Unresolved peaks.

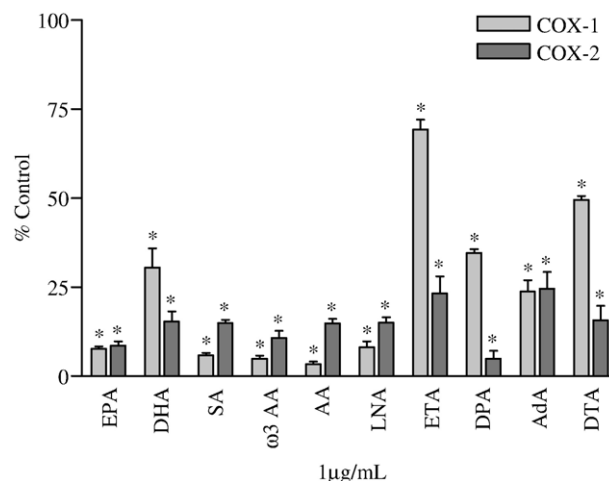


Fig. 6. Inhibition of COX-1 and COX-2 by PUFA from Lyprinol (mean \pm SEM, $n=3$). *Significantly different from methanol control based on one way ANOVA with Dunnett's post test ($p<0.05$). Eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA); stearidonic acid (SA); ω 3 arachidonic acid (ω 3 AA); ω 6 arachidonic acid (AA); linolenic acid (LNA); eicosatrienoic acid (ETA); docosapentaenoic acid (DPA); docosatrenoic acid (AdA, adrenic acid); docosatrenoic acid (DTA).

conclusively. The presence of arachidonic acid in fraction 2 ($R_t=9$ min) indicated that not all the substrate was utilised in the enzyme reaction. The COX-2 reaction on arachidonic acid displayed a similar metabolite profile to the COX-1 reaction, however it appeared less abundant in prostaglandin product (Fig. 8).

Incubation of Lyprinol with COX-1 (Fig. 9) and COX-2 (Fig. 10) also indicated the presence of prostaglandin metabolites in fractions 3 and 4, despite the fact there was no addition of exogenous arachidonic acid. The prostaglandin metabolites were less evident in the COX-2 Lyprinol reaction compared to the COX-1 Lyprinol reaction. In addition, fraction 2 from the Lyprinol reactions (COX-1 and COX-2) indicated non-arachidonate-derived fatty acid esters eluting in the customary arachidonic acid ester region (R_t range 8–9 min).

4. Discussion

Marine organisms such as the bivalve mussels examined in this study contain an abundance of bioactive lipids. Bioactive lipids can include fatty acids, sphingolipids, phytosterols, diacylglycerols, diterpenes, sesquiterpenes and saponins; and many of these can influence human health and disease (Glaser and Lock, 1995; Huynh et al., 1997; Li and Sinclair, 2002). Mussels, like other marine organisms such as oily fish, are abundant in ω 3 PUFA, particularly EPA and DHA (Joseph, 1982; King et al., 1990). Marine oils have been linked to alleviating the symptoms of inflammatory conditions, such as arthritis, skin disorders and asthma (Calder, 2001), as well as reducing certain risk factors of cardiovascular disease (Herold and Kinsella, 1986; Paganelli et al., 2001).

Lipid analysis of Hallam Cove *P. canaliculus* used in this study reveals a complex mixture of marine PUFA (comprising 45% of total lipid), including high concentrations of EPA (13%)

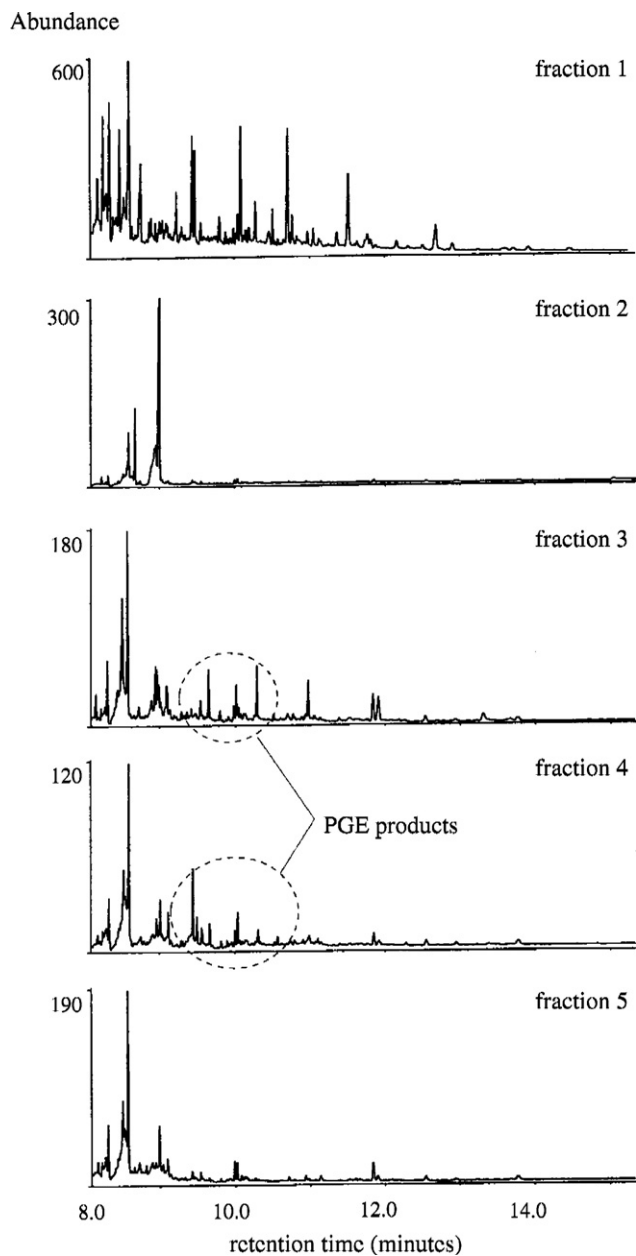


Fig. 7. GCMS analysis of metabolites produced from the enzyme reaction of COX-1 on arachidonic acid.

al., 1985; Lubet et al., 1985; Marsden and Weatherhead, 1998; Okumus and Stirling, 1998; Hawkins et al., 1999; James et al., 2001; Freitas et al., 2002). The mussel species tested here are sampled from the same location at the same time, therefore it is possible that the comparatively higher lipid content of *P. canaliculus* is species specific, rather than related to environmental factors. Apart from the higher lipid composition in *P. canaliculus*, there are no overall significant differences between the two mussel species in lipid class composition. Triglycerides, sterols and phospholipids are the main component lipids, and there is modest free fatty acid content (Murphy et al., 2002).

The complex mixture of long chain PUFA of *P. canaliculus* is thought to contribute to its anti-inflammatory activity. These PUFA are enriched 20 fold in the Lyprinol extract of the dried

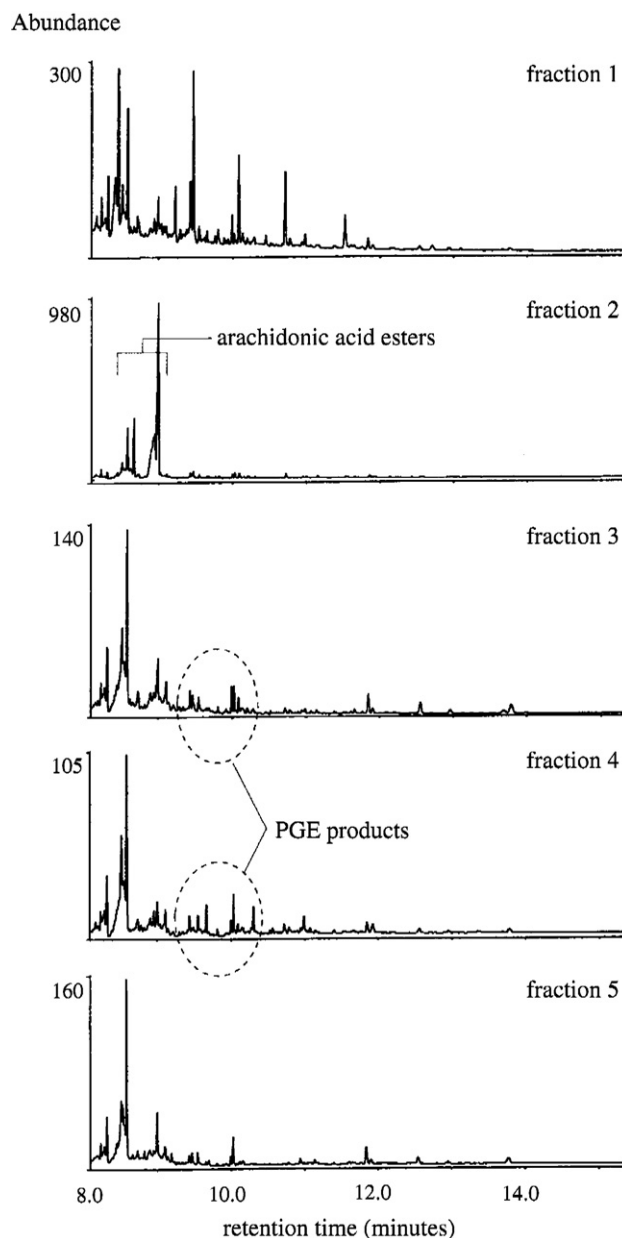


Fig. 8. GCMS analysis of metabolites produced from the enzyme reaction of COX-2 on arachidonic acid.

and DHA (21%) (Murphy et al., 2003). These PUFA are obtained through the food chain, in particular from phytoplankton which are also high in ω 3 PUFA (Budge et al., 2001). Sterol analysis shows cholesterol to be the predominant sterol in the mussel, and there is also a variety of other sterols present, including phytosterols (Murphy et al., 2002). Hallam Cove *P. canaliculus* exhibit no significant difference in PUFA or sterol composition when compared to Hallam Cove *M. edulis*, however *P. canaliculus* has a significantly higher total lipid content on a dry weight basis (7.67% compared to 6.00% for *M. edulis*) (Murphy et al., 2003). The lipid content of marine molluscs reportedly varies with season, life cycle and habitat; and biochemical changes may also result from variability in metabolic activity, location, sex and spawning (Kluytmans et

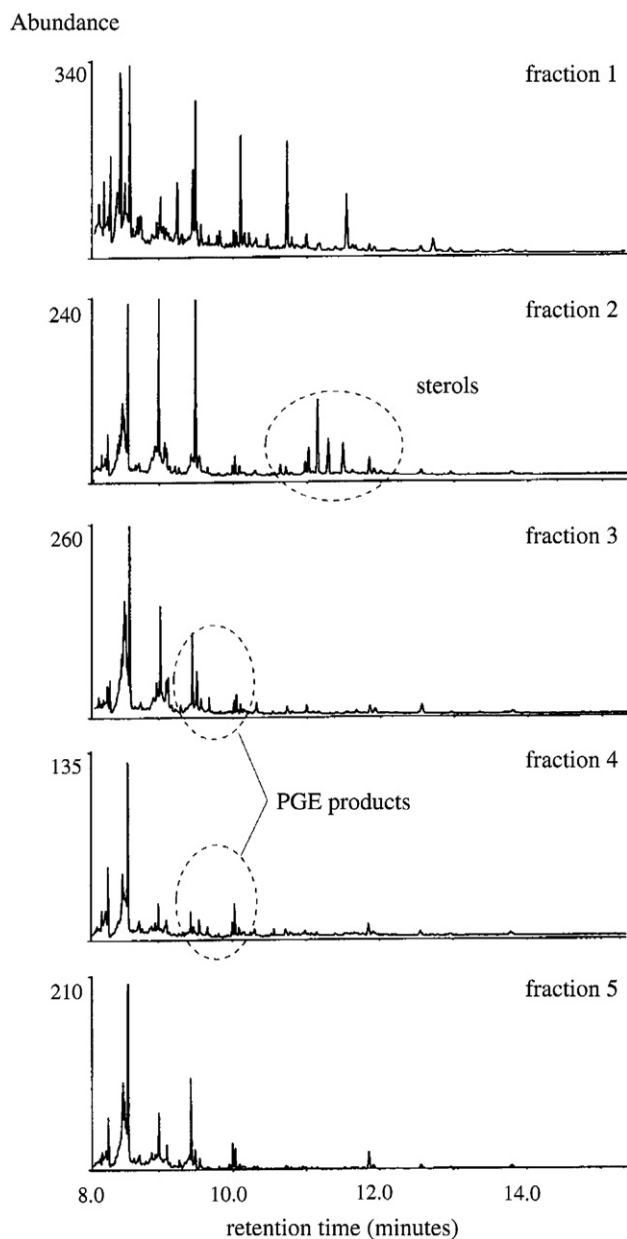


Fig. 9. GCMS analysis of metabolites produced from the enzyme reaction of COX-1 on Lyprinol.

mussel, which is a direct result of the supercritical fluid extraction procedure used to extract the lipid (Whitehouse et al., 1997). Free fatty acids (54%) and triglycerides (27%) constitute the predominant lipid classes of Lyprinol. This composition makes it a very different lipid extract compared to Blackmores fish oil, which is composed mainly of triglyceride (30%) (www.blackmores.com.au). Further investigation of Lyprinol by silver-ion TLC and GCMS reveals a unique mixture of PUFA including EPA (20:5 ω -3), DHA (22:6 ω -3), SA (18:4 ω -3), ETA (20:3), DTA (22:3 ω -3), α -LNA(18:3 ω -3) and the rare ω 3-AA (20:4 ω -3). In comparison, Blackmores fish oil, which is sourced from oily cold water fish and which may contain anchovy, sardines, salmon and mackerel, is standardised to contain EPA (18%) and DHA (12%) in triglyceride form.

The COX inhibition assay used in this study is based on the oxidation of 1-DCF by the hydroperoxide formed in the cyclooxygenase reaction. The method measures the second peroxidase reaction of the enzyme which has a higher turnover than the initial oxygenase reaction; the oxygenase reaction is considered the limiting step in the overall rate of the enzyme (Larsen et al., 1996). The *P. canaliculus* and *M. edulis* total lipid extracts examined here exhibited little COX inhibitory activity until saponified. The greater inhibition of COX upon saponification has indicated that the free fatty acid fraction is likely to be in part responsible for the anti-COX activity. ω 3 Polyunsaturated fatty acids are known to competitively inhibit the COX enzyme (Larsen et al., 1996), and it is likely

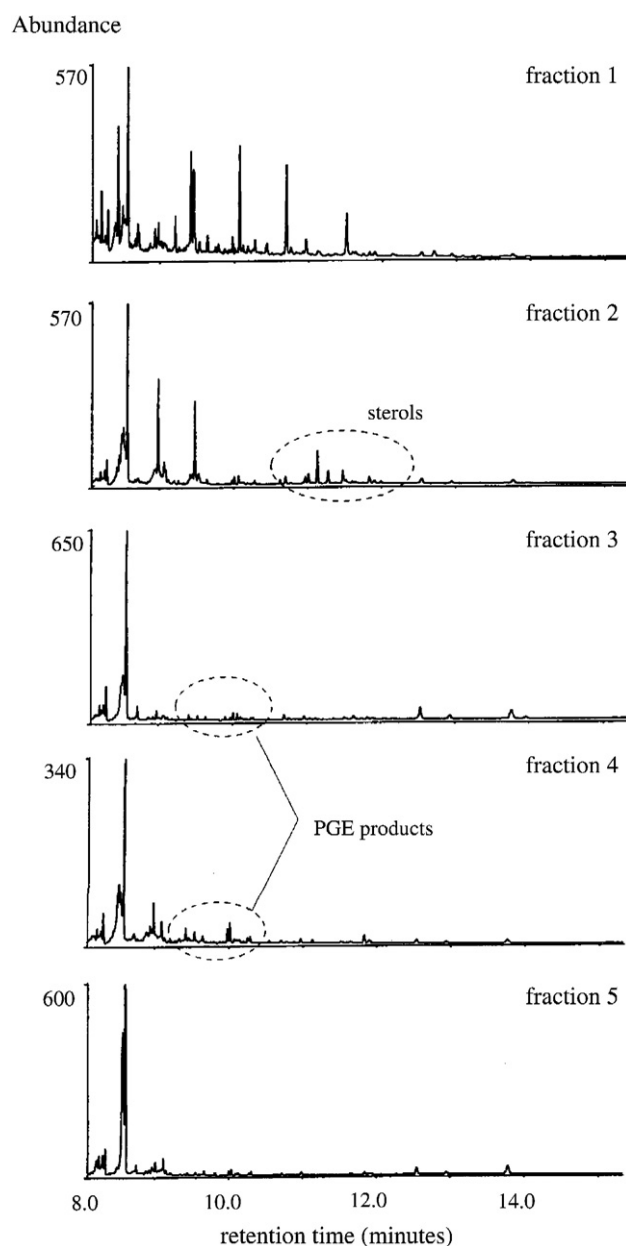


Fig. 10. GCMS analysis of metabolites produced from the enzyme reaction of COX-2 on Lyprinol.

that these PUFA contributed to the anti-COX activity of the saponified fractions.

Lyprinol and hydrolysed Lyprinol exhibited strong inhibition of both COX-1 and COX-2, and did not appear to exhibit selectivity in their inhibition of the two enzymes. There was little difference in inhibitory activity between Lyprinol and hydrolysed Lyprinol when tested at higher concentrations (100 and 1 $\mu\text{g}/\text{mL}$), however, when examining IC_{50} values, hydrolysed Lyprinol has a 10-fold lower IC_{50} value than for Lyprinol. The high percentage of free fatty acids in the Lyprinol extracts supports the argument that the free fatty acid fraction is exerting the inhibitory effect. The enzyme-treated protease and protease–lipase *P. canaliculus* lipid extracts exhibit a similar level of inhibition compared to Lyprinol and hydrolysed Lyprinol at the same concentration, suggesting they contain similar levels of bioactive compounds. Again, it is likely to be the PUFA component that is biologically active; as this conclusion is further indicated by the stronger inhibition exhibited by the PL extract compared to the P extract. The enzyme-assisted solvent extraction of *P. canaliculus* mussel meat utilises a proteolytic enzyme to facilitate oil release, and a lipase enzyme to increase the yield of the highly bioactive free fatty acid fraction of *P. canaliculus*.

Fish oil at both 100 $\mu\text{g}/\text{mL}$ and 1 $\mu\text{g}/\text{mL}$ exhibited less COX inhibitory activity compared to Lyprinol at the same concentrations. Upon hydrolysis, fish oil exhibited similar inhibition at 1 $\mu\text{g}/\text{mL}$ to Lyprinol and hydrolysed Lyprinol. Fish oil is predominantly composed of triglyceride molecules which are rich in EPA and DHA, while Lyprinol is a more complex mixture of lipid classes and is particularly high in free fatty acids. Seemingly, hydrolysis of fish oil results in the release of inhibitory PUFA giving it a similar activity to Lyprinol.

There are at least two isoforms of mammalian COX, the constitutive COX-1 enzyme, and COX-2 which is induced by inflammatory stimuli. Although inhibition of COX-2 is beneficial to the reduction of the inflammatory response, there is debate concerning the safe usage of NSAIDs and selective COX-2 inhibitors as anti-inflammatory agents in relation to gastrointestinal and cardiovascular events (Parente and Perretti, 2003). In the present study, Lyprinol and the lipid extracts from *P. canaliculus* exhibited no inhibition selectivity or preference for either COX-1 or COX-2, and this was apparent for the extracts at various concentrations. This observation is in contrast to the NSAID indomethacin, which is a non-selective inhibitor of COX that shows preferential activity against COX-1 (Hull et al., 2003). At comparable concentrations, Lyprinol is as effective as Indomethacin at inhibition of COX-1, but is much more effective at inhibition of COX-2.

Upon separating Lyprinol into its constituent lipid classes, the free fatty acid fraction exhibited the highest inhibition of the Lyprinol lipid classes, and it is likely that the unique mixture of ω -3 PUFA in Lyprinol, including EPA and DHA contained within this fraction, act directly as competitive substrate inhibitors of AA metabolism by COX. The triglyceride fraction of Lyprinol also exhibited some inhibitory activity, but the mechanism of action for this is unclear. In most marine organisms the majority of fatty acids occur predominantly as

phospholipids (Volkman et al., 1998), and then as triglycerides. Triglycerides are energy storage lipids and can be used as a condition index for marine organisms (Parrish et al., 2000).

Sterol compounds can be highly bioactive, and various sterols have been known to influence human health and disease, the most cited example being cholesterol which has a harmful effect on cardiovascular health (Patel and Thompson, 2006). Cholesterol is identified as the major sterol in both *P. canaliculus* and *M. edulis* (Murphy et al., 2003), and this has some implications when considering the mussel preparations as a dietary treatment. Altogether, 18 individual sterols are identified in *P. canaliculus* and *M. edulis*, with desmosterol/brassicasterol (coeluting), campesterol, *trans*-22-dehydrocholesterol and 24-nordehydrocholesterol accounting for over 65% of the remaining sterol fraction (Murphy et al., 2002). Phytosterols, such as β -sitosterol which is also reported in *P. canaliculus*, are known to generate anti-inflammatory activity in acute animal inflammation models (Li and Sinclair, 2002). In this study, the sterol and sterol ester fractions exhibited no significant inhibition of COX-1, although there was some inhibition of COX-2. The reason for this selectivity is unclear. It is possible that certain sterol compounds, including phytosterols derived from the mussel's phytoplankton diet, exhibit a positive, albeit small, effect on COX-2 inhibition.

The phospholipids (polar lipid) fraction of Lyprinol also exhibited moderate inhibition of COX-2, while COX-1 inhibition was not significant. Phospholipid molecules can be highly bioactive and may contribute to the COX-2 inhibition exhibited (Eligini et al., 2002). Furthermore, there could be a variety of other polar compounds (oxidised PUFA, glycolipids, partially hydrolysed lysophospholipids and lysoglycolipids) in the fraction that may be responsible for the anti-COX activity seen.

In relation to the purified PUFA tested in this study, SA, ω 3-AA, α -LNA and EPA all exhibited high inhibition of both COX isoforms. Certain PUFA are known to inhibit COX by acting as competitive substrate inhibitors. For example, EPA is known to be a substrate, albeit a poorer one than AA, for prostaglandin biosynthesis (Corey et al., 1983). Also, research has shown that prostaglandins of the ω 3-series, derived from EPA, are less biologically active, and ω 3-AA has been shown to be as potent as EPA in inhibiting COX (Croset et al., 1999). Although not as active as the above mentioned PUFA, the 22 carbon length fatty acids, AdA, DHA, and DPA exhibited high inhibition of the COX isoforms in this study. Of interest, DHA and DPA exhibited higher inhibition of COX-2 compared to COX-1. Docosahexaenoic acid is not an alternate substrate for mammalian COX but it is reported to be a potent competitive inhibitor of the enzyme (Corey et al., 1983). Also, DPA is shown to inhibit or influence the activity of COX in various experiment models (Akiba et al., 2000). Eicosatrienoic acid and DTA, meanwhile, only exhibit moderate inhibition of COX-1, although inhibition of COX-2 is still strong. These results indicate that the three bond configuration of these PUFA is not greatly effective at inhibiting COX-1, although they are still highly active in COX-2 inhibition, and this is likely to be due to the larger COX-2 active site that can accommodate PUFA

substrates more readily (Vane et al., 1998). Significant inhibitory effect is also shown by ω 6-AA, when pre-incubated for 5 min with both COX-1 and COX-2. This observed inhibition may be attributed to a possible autocatalysed self-inactivation of the COX enzyme in the presence of excess arachidonic acid substrate (Larsen et al., 1996). It is also possible that the inhibition observed by the various bioactive PUFA occurs via a similar mechanism.

The present investigation of prostaglandin metabolite production by COX employed an *in vitro* enzyme system, which aimed to simplify the investigation of the Lyprinol interaction with COX by eliminating further metabolism by other enzymes in the arachidonic cascade, which would otherwise occur in a whole cell or *in vivo* assay. The pure enzyme reaction studies, as discussed above, have shown Lyprinol to inhibit the turnover reaction of AA by the COX isoforms. However, when Lyprinol without the presence of AA is incubated with COX, the GCMS results also indicate the presence of prostaglandin metabolites, suggesting that alternate PUFA substrates from Lyprinol are being converted to prostaglandin analogues. This designates the mechanism of inhibition by Lyprinol as that of a competitive substrate inhibitor. Lyprinol, like other marine oils, is high in ω 3 PUFA such as EPA and DHA. Studies have shown EPA-derived prostaglandin metabolites to be produced via the COX reaction, and these metabolites are considered to be less biologically active than their AA counterparts (Corey et al., 1983). Docosahexaenoic is also reported to be a substrate for COX (Larsen et al., 1996). It follows that other Lyprinol PUFA could be metabolised to alternate prostaglandin metabolites with unknown biological actions in different cell types. For example, ω 3-AA is not known to be converted into prostaglandins by COX, however platelet COX is shown to produce 12-hydroxy-8,11,14-heptadecatrienoic acid from this PUFA (Croset et al., 1999). For these Lyprinol reactions, it is noted that the prostaglandin metabolite peaks are relatively abundant indicating that alternate PUFA substrate is not metabolised at a slower rate than AA. This is in contrast to literature which reports that the PUFA substrates, α -LNA, AdA and EPA, are substrates for COX with decreasing reaction rates in the mentioned order, behind AA (Larsen et al., 1996). However the long incubation time of 15 min for each reaction may result in a defined metabolite concentration before auto-catalysed inactivation of the COX enzyme occurs.

There has been debate regarding the bioactive components of *P. canaliculus*. Many researchers believe it to reside in the lipid component of the mussel (Whitehouse et al., 1997; Gibson and Gibson, 1998), while other groups have reported the protein (Couch et al., 1982; Mani et al., 1998) and glycogen (Miller et al., 1993; Tan and Berridge, 2000) fractions to be efficacious. Of importance in these clinical trials is the fact that Seatone and Lyprinol have shown to be well tolerated, with virtually no adverse side effects (Rainsford and Whitehouse, 1980; Whitehouse et al., 1997; Shiels and Whitehouse, 2000), and this is in contrast to traditional anti-inflammatory treatments such as NSAIDs and the more recent COX-2 specific inhibitors, which can have adverse effects

especially when taken for long periods of time. Lyprinol and particular fractions from *P. canaliculus* that are high in PUFA are shown in this study to exhibit strong inhibition of the COX isoenzymes, and are postulated to do so by acting as alternate substrates for these enzymes. These results support the use of the commercial mussel extracts, in particular Lyprinol, as an alternative for conventional NSAIDs and fish oil treatment in the relief of the symptoms of arthritis.

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References

- Akiba, S., Murata, T., Kitatani, K., Sato, T., 2000. Involvement of lipoxygenase pathway in docosapentaenoic acid-induced inhibition of platelet aggregation. *Biol. Pharm. Bull.* 23, 1293–1297.
- Bligh, E., Dyer, 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917.
- Budge, S.M., Parrish, C.C., McKenzie, C.H., 2001. Fatty acid composition of phytoplankton, settling particulate matter and sediments at a sheltered bivalve aquaculture site. *Mar. Chem.* 76, 285–303.
- Calder, P.C., 2001. N-3 polyunsaturated fatty acids, inflammation and immunity: pouring oil on troubled waters or another fishy tale? *Nutr. Res.* 21, 309–341.
- Charlier, C., Michaux, C., 2003. Dual inhibition of cyclooxygenase-2 (COX-2) and 5 lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs. *Eur. J. Med. Chem.* 38, 645–659.
- Christie, W.W., 1989. *Gas Chromatography and Lipids: A Practical Guide*. The Oily Press, Scotland.
- Corey, E.J., Shih, C., Cashman, J.R., 1983. Docosahexaenoic acid is a strong inhibitor of prostaglandin but not leukotriene biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* 80, 3581–3584.
- Couch, R.A.F., Ormrod, D.J., Miller, T.E., Watkins, W.B., 1982. Anti-inflammatory activity in fractionated extracts of the green-lipped mussel. *N.Z. Med. J.* 95, 803–806.
- Croset, M., Bordet, J.C., Lagarde, M., 1999. Inhibition of prostaglandin H synthase and activation of 12-lipoxygenase by 8,11,14,17-eicosatetraenoic acid in human endothelial cells and platelets. *Biochem. Pharmacol.* 57, 631–638.
- Dugas, B., 2000. Lyprinol® inhibits LTB₄ production by human monocytes. *Allerg. Immunol.* 32, 284–289.
- Eligini, S., Brambilla, M., Banfi, C., Camera, M., Sironi, L., Barbieri, S.S., Auwerx, J., Tremoli, E., Colli, S., 2002. Oxidized phospholipids inhibit cyclooxygenase-2 in human macrophages via nuclear factor- κ B/I κ B- and ERK-dependent mechanisms. *Cardiovasc. Res.* 55, 406–415.
- Freites, L., Fernandez-Reiriz, M.J., Labarta, U., 2002. Fatty acid profiles of *Mytilus galloprovincialis* (Lmk) mussel of subtidal and rocky shore origin. *Comp. Biochem. Physiol., Part B Biochem. Mol. Biol.* 132, 453–461.
- Gibson, S.L.M., Gibson, R.G., 1998. The treatment of arthritis with a lipid extract of *Perna canaliculus*: a randomized trial. *Complement. Ther. Med.* 6, 122–126.
- Gibson, R.G., Gibson, S.L.M., Conway, V., Chappel, D., 1980. *Perna canaliculus* in the treatment of arthritis. *Practitioner* 224, 955–960.
- Glaser, K.B., Lock, Y.W., 1995. Regulation of prostaglandin H synthase 2 expression in human monocytes by the marine natural products manoalide and scalarial: novel effects independent of inhibition of lipid mediator production. *Biochem. Pharmacol.* 50, 913–922.
- Hawkins, A.J.S., James, M.R., Hickman, R.W., Hatton, S., Weatherhead, M., 1999. Modelling of suspension-feeding and growth in the green-lipped mussel *Perna canaliculus* exposed to natural and experimental variations of

- season availability in the Marlborough Sounds, New Zealand. MEPS 191, 217–232.
- Herold, P.M., Kinsella, J.E., 1986. Fish oil consumption and decreased risk of cardiovascular disease: a comparison of findings from animal and human feeding trials. *Am. J. Clin. Nutr.* 43, 566–598.
- Hull, M.A., Gardner, S.H., Hawcroft, G., 2003. Activity of the non-steroidal anti-inflammatory drug indomethacin against colorectal cancer. *Cancer Treat. Rev.* 29, 309–320.
- Huynh, C., Pinelli, E., Puiseux-Dao, S., Pfohl-Leszkowicz, A., 1997. Okadaic acid stimulates phospholipase A2, cyclooxygenase and lipoxygenase. *Mutat. Res. Fundam. Mol. Mech. Mutagenesis* 379, S53.
- James, M.R., Weatherhead, M.A., Ross, A.H., 2001. Size-specific clearance, excretion, and respiration rates, and phytoplankton selectivity for the mussel *Perna canaliculus* at low levels of natural food. *N.Z. J. Mar. Freshw. Res.* 35, 73–86.
- Joseph, J.D., 1982. Lipid composition of marine and estuarine invertebrates. Part II: mollusca. *Prog. Lipid Res.* 21, 109–153.
- King, I., Childs, M.T., Dorsett, C., Ostrander, J.G., Monsen, E.R., 1990. Shellfish: proximate composition, minerals, fatty acids, and sterols. *J. Am. Diet. Assoc.* 90, 677–685.
- Kluytmans, J.H., Boot, J.H., Oudejans, R.C.H.M., Zandee, D.I., 1985. Fatty acid synthesis in relation to gametogenesis in the mussel *Mytilus edulis* L. *Comp. Biochem. Physiol.*, B 81, 959–963.
- Kosuge, T., Sugiyama, K., 1989. Stabilized mussel extract. United States Patent 4801453.
- Kosuge, T., Tsuji, K., Ishida, H., Yamaguchi, T., 1986. Isolation of an anti-histaminic substance from green-lipped mussel (*Perna canaliculus*). *Chem. Pharm. Bull.* 34, 4825–4828.
- Larsen, L.N., Dahl, E., Bremer, J., 1996. Peroxidative oxidation of leuco-dichlorofluorescein by prostaglandin H synthase in prostaglandin biosynthesis from polyunsaturated fatty acids. *Biochim. Biophys. Acta* 1299, 47–53.
- Li, D., Sinclair, A.J., 2002. Macronutrient innovations: the role of fats and sterols in human health. *Asia Pac. J. Clin. Nutr.* 11, S155–S162.
- Lubet, P., Brichon, G., Besnard, J.Y., Zwingelstein, G., 1985. Composition and metabolism of lipids in some tissues of the mussel *Mytilus galloprovincialis* L. (Moll. bivalvia) — *in vivo* and *in vitro* incorporation of 1(3)-[³H]-glycerol. *Comp. Biochem. Physiol.*, B 82, 425–431.
- Macrides, T.A., Kalafatis, N., 2000. Super-critical lipid extract from mussels having anti-inflammatory activity. United States Patent 6083536.
- Mani, S., Whiteside, J.F., Lawson, J.W., 1998. Use of perna and dimethylglycine (DMG) as immunotherapeutic agents in autoimmune disease and melanoma. *Crit. Rev. Biomed. Eng.* 26, 405–406.
- Marsden, I.D., Weatherhead, M.A., 1998. Effects of aerial exposure on oxygen consumption by the New Zealand mussel *Perna canaliculus* (Gmelin, 1791) from an intertidal habitat. *J. Exp. Mar. Biol. Ecol.* 230, 15–29.
- Miller, T.E., Ormrod, D.J., 1980. The anti-inflammatory activity of *Perna canaliculus* (NZ green-lipped mussel). *N.Z. Med. J.* 667, 187–193.
- Miller, T.E., Dodd, J., Ormrod, D.J., Geddes, R., 1993. Anti-inflammatory activity of glycogen extracted from *Perna canaliculus* (NZ green-lipped mussel). *Inflamm. Res.* 38, C139–C142.
- Murphy, K.J., Mooney, B.D., Mann, N.J., Nichols, P.D., Sinclair, A.J., 2002. Lipid, FA, and sterol composition of New Zealand green lipped mussel (*Perna canaliculus*) and Tasmanian blue mussel (*Mytilus edulis*). *Lipids* 37, 587–595.
- Murphy, K.J., Mann, N.J., Sinclair, A.J., 2003. Fatty acid and sterol composition of frozen and freeze-dried New Zealand green lipped mussel (*Perna canaliculus*) from three sites in New Zealand. *Asia Pac. J. Clin. Nutr.* 12, 50–60.
- Okumus, I., Stirling, H.P., 1998. Seasonal variations in the meat weight, condition index and biochemical composition of mussels (*Mytilus edulis* L.) in suspended culture in two Scottish sea lochs. *Aquaculture* 159, 249–261.
- Paganelli, F., Maixent, J.M., Duran, M.J., Parhizgar, R., Pironi, G., Sennoune, S., 2001. Altered erythrocyte *n*-3 fatty acids in Mediterranean patients with coronary artery disease. *Int. J. Cardiol.* 78, 27–32.
- Parente, L., Perretti, M., 2003. Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. *Biochem. Pharmacol.* 65, 153–159.
- Parrish, C.C., Abrajano, T.A., Budge, S.M., Helleur, R.J., Hudson, E.D., Pulchan, K., Ramos, C., 2000. Lipid and phenolic biomarkers in marine ecosystems: analysis and applications. In: Wangersky, P. (Ed.), *The Handbook of Environmental Chemistry. Marine Chemistry*, vol. 5. Springer-Verlag, Berlin.
- Patel, M.D., Thompson, P.D., 2006. Phytosterols and vascular disease. *Atherosclerosis* 186, 12–19.
- Rainsford, K.D., Whitehouse, M.W., 1980. Gastroprotective and anti-inflammatory properties of green lipped mussel (*Perna canaliculus*) preparation. *Arzneim.-Forsch.* 30, 2128–2132.
- Shiels, I.A., Whitehouse, M.W., 2000. Lyprinol: anti-inflammatory and uterine-relaxant activities in rats, with special reference to a model for dysmenorrhea. *Allergie Immunol.* 32, 279–283.
- Tan, A.S., Berridge, M.V., 2000. Superoxide produced by activated neutrophils efficiently reduces the tetrazolium salt, WST-1 to produce a soluble formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening anti-inflammatory agents. *J. Immunol. Methods* 238, 59–68.
- Vane, J.R., Bakhle, Y.S., Botting, R.M., 1998. Cyclooxygenases 1 and 2. *Annu. Rev. Pharmacol. Toxicol.* 38, 97–120.
- Volkman, J.K., Barrett, S.M., Blackburn, S.I., Mansour, M.P., Sikes, E.L., Gelin, F., 1998. Microalgal biomarkers: a review of recent research developments. *Org. Geochem.* 29, 1163–1179.
- Whitehouse, M.W., Macrides, T.A., Kalafatis, N., Betts, W.H., Haynes, D.R., Broadbent, J., 1997. Anti-inflammatory activity of a lipid fraction (Lyprinol) from the NZ green-lipped mussel. *Inflammopharmacology* 5, 237–246.