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In vitro anti-thrombotic and anti-coagulant properties of blacklip abalone (*Haliotis rubra*) viscera hydrolysate

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Abstract Abalone viscera contain sulphated polysaccharides with anti-thrombotic and anti-coagulant activities. In this study, a hydrolysate was prepared from blacklip abalone (*Haliotis rubra*) viscera using papain and bromelain and fractionated using ion exchange and size exclusion chromatography. Hydrolysates and fractions were investigated for in vitro thrombin inhibition mediated through heparin cofactor II (HCII) as well as anti-coagulant activity in plasma and whole blood. On the basis of sulphated polysaccharide concentration, the hydrolysate inhibited thrombin through HCII with an inhibitor concentration at 50% (IC50) of 16.5 μg/mL compared with 2.1 μg/mL for standard heparin. Fractionation concentrated HCII-mediated thrombin inhibition down to an IC50 of 1.8 μg/mL and improved anti-coagulant activities by significantly delaying clotting time. This study confirmed the presence of anti-thrombotic and anti-coagulant molecules in blacklip abalone viscera and demonstrated that these activities can be enriched with a simple chromatography regime. Blacklip abalone viscera warrant further investigation as a source of nutraceutical or functional food ingredients.

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Introduction

Abalone, an edible marine gastropod, contains a variety of bioactive compounds with anti-oxidant, anti-thrombotic, anti-inflammatory, anti-microbial and anti-cancer activities [1]. For thousands of years, different cultures have consumed abalone as a traditional functional food believing consumption provides health benefits [2]. Abalone in both raw and processed form has gained very high market value in countries like China and Singapore [3]. The preparation of abalone for sale as a food product generates a disproportionate amount of waste with only one third of the animal, the foot, deemed marketable. The remaining two thirds of the animal, comprised of the shell and viscera, is not marketable and is usually discarded. Abalone viscera are considered inedible by producers as well as customers; therefore, this visceral matter, accounting for 15–25%, is normally discarded as industrial waste [4]. Amongst the abalone processing waste products of trimmings, shells and viscera, many studies have focussed on viscera as a source of bioactive compounds for use as ingredients in nutraceuticals and functional foods.

Considerable research has been performed on abalone viscera as a source of anti-thrombotic molecules where sulphated polysaccharides have been extracted from abalone and assessed for in vitro anti-coagulant activity measured through the prolongation of activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT). Studies have demonstrated disk abalone (*Haliotis discus hannai* Ino) viscera, gonads and pleopods to be sources of potent anti-thrombotic and anti-coagulant polysaccharides, highlighting the therapeutic potential of abalone in coagulation control [5]. According to Li et al. [6], aqueous extracts of disk abalone viscera improve PT, aPTT and TT but require two- to fivefold higher concentrations (on a sulphated polysaccharide basis) to maintain the same activity as heparin (a

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widely used anti-thrombotic molecule and common assay control). In similar separation studies, different types of sulphated polysaccharides have been obtained from visceral portions and gonads of abalone [7]. Wang et al. [8] isolated and characterised three anti-thrombotic sulphated polysaccharides from the pleopods and viscera of disk abalone.

Previous studies involving an extract prepared from processed blacklip abalone viscera (canned and liquid abalone waste products) using different proteolytic enzymes followed by anion exchange chromatography also showed significant anti-thrombotic and anti-coagulant activity. In vitro PT and aPTT were prolonged by all abalone extracts with PT increasing in response to anionic fractions. A similar trend was observed in the aPTT in vitro assay [9]. Overall, many studies have concluded that abalone extracts are an important source of anti-thrombotic and anti-coagulant molecules; however, purification is required prior to use as an ingredient in nutraceuticals or functional foods.

In this study, hydrolysates were prepared from blacklip abalone (*Haliotis rubra*) and fractionated into pools of molecules enriched with sulphated polysaccharides with enhanced anti-thrombotic and anti-coagulant activities. In vitro anti-thrombotic activity was measured through heparin cofactor II (HCII), while in vitro anti-coagulant activity was measured in plasma and whole blood using PT, aPTT and thromboelastography (TEG) assays.

Material and methods

Preparation of hydrolysate

Wild caught Australian blacklip abalone (*H. rubra*) visceral samples were provided by Tasmanian Seafoods, Hobart, Australia. Hydrolysates were prepared using 5.0% *w/w* food grade 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 prepared according to the method described by Suleria et al. [9].

Estimation of sulphated polysaccharide content

Absorbance from all colorimetric assays was measured using a Spectra-Max M3 System spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at the nominated wavelength. Concentrations for samples, standards and controls were determined and the associated instrument software (SoftMax-Pro 6.1).

Dimethylmethylene blue assay

Sulphated polysaccharide concentration was initially estimated in all samples through interaction with 1,9-dimethylmethylene blue dye (DMMB, Sigma-Aldrich, Castle Hill, NSW, Australia) by following the method of Suleria et al. [9].

Blyscan sulphated glycosaminoglycan assay

Sulphated polysaccharide concentration was measured in all samples and pooled fractions using the Blyscan™ Sulphated Glycosaminoglycan assay according to manufacturer's instructions (Biocolor Ltd., Carrickfergus, County Antrim, UK) with modifications of Suleria et al. [9].

Estimation of protein content

Protein content was estimated in all samples in triplicate 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. Absorbance was measured at 562 nm.

Estimation of total neutral sugars

The total neutral sugar assay was based on the phenol-sulphuric acid method where polysaccharides are hydrolysed and converted into furfural and hydroxylfurfural [10]. This was achieved by adding 150 μL concentrated sulphuric acid to 50-μL sample or glucose standard in a 96-well plate in triplicate, followed by 500 rpm orbital shaking at room temperature for 1 min in a fume hood. A solution of 5% *v/v* phenol in water (30 μL) was added to each well before being heated to 90 °C and mixed using 300 rpm orbital shaking for 30 min. The 96-well plate was allowed to cool at room temperature for 5 min before absorbance was measured at 490 nm. GraphPad Prism 5 Software for Windows (GraphPad, San Diego, CA, USA, www.graphpad.com) was used to extrapolate the neutral sugar concentration in the abalone samples from the glucose standard curve.

Separation of blacklip abalone hydrolysate using AEC-FPLC

Q Sepharose™ Big Beads (372.5 mL) were packed into an empty GE-XK 26/70 column (700 mm × 26 mm, GE Healthcare Life Sciences, 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 deionised water (buffer A) before a sample of approximately 800–900 mg, on a sulphated polysaccharide basis,

was loaded onto the column. Flow rate was set at 5 mL/min with a limit column pressure of 1 MPa. After isocratic washes of buffer A over 11 h (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 [11] and 206 nm for glycosaminoglycan detection [12]. Conductivity was monitored throughout the run also. The collected fractions were pooled on the basis of their interactions with 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 as described above.

Separation of AEC pools by SEC-FPLC

Sephacryl® S-100 HR Beads (230 mL) was packed into an empty GE-XK 16/100 column (1000 mm × 16 mm, GE Healthcare Life Sciences, Chicago, IL, USA). The packed column was attached to a FPLC system (ÄKTA Lab-Scale Systems, GE Healthcare Life Sciences, Chicago, IL, USA). The column was equilibrated with deionised water before a sample of approximately 2 mL (8–10 mg sulphated polysaccharides as estimated by the DMMB assay) was loaded onto the column. Flow rate was set at 0.5 mL/min with a limit column pressure of 1 MPa. Fractions (10 mL) were collected using isocratic elution at 0.2 M NaCl over 20 h. Elution was monitored at 280 nm for protein content, 240 nm for peptide bond absorption and 206 nm for non-specific carbohydrate detection. Fractions were collected and pooled into four size exclusion chromatography (SEC) pools (denoted SEC pools A, A*, B and C) on the basis of their sulphated polysaccharide concentration (using the DMMB assay). For further analysis, all pooled samples were desalted as described above.

Assessment of anti-thrombotic and anti-coagulant activity

Heparin cofactor II-mediated thrombin inhibition assay

In vitro HCII-mediated thrombin inhibition was measured in all samples using a kinetic assay as previously described [13] with modifications [9, 14]. Inhibitor concentration at 50% (IC50) values were calculated from the percentage inhibition using GraphPad Prism 5 (using variable slope response curves (four parameters)) and expressed as IC50 (with all R^2 above 0.96) with a 95% confidence interval.

Prothrombin time assay

To measure PT, 100 µL citrated plasma was added to a glass clotting tube and incubated at 37 °C in a heating block of a Hyland-Clotek clotting machine (Hyland, USA) for 5 min. Volumes of 50 µL of saline (negative control), heparin/Clexane (positive control, Sigma-Aldrich) or sample were added to the tube. The volume was then adjusted to 150 µL with citrated plasma before the final addition of 100-µL Thromborel S® (Dade Behring Inc. Newark, USA, international sensitivity index (ISI) = 1) to initiate clotting [9]. Time in seconds until clot formation was measured in triplicate by the clotting machine and expressed as the mean ± standard error. For comparison, the international normalised ratio (INR) was calculated for each PT assay as below:

$$\text{INR} = \left[\text{PT sample}\Big/\text{PT saline control}\right]^{\text{ISI}}$$

The INR is used to monitor individuals who are being treated with an anti-coagulant. The therapeutic range of INR is normally 2.0–3.0 [15].

Activated partial thromboplastin time assay

To measure aPTT, 100 µL citrated plasma, 100 µL Triniclot (Haemostasis, Wicklow, Ireland, ISI = 1–1.3) and diluted abalone hydrolysate and AEC pooled fractions were added to a clotting tube. The final volume was adjusted to 250 µL with saline. The clotting tube was incubated at 37 °C in a Hyland-Clotek clotting machine for 5 min before 50 µL 50 mM CaCl$_2$ was added to initiate clotting [9]. Time in seconds until clot formation was measured in triplicate and expressed as the mean ± standard error. For comparison, the INR was calculated for each aPTT assay using an ISI of 1.2.

Thromboelastography

To measure clot dynamics in the presence of the abalone hydrolysate and AEC pooled fractions, TEG (Haemonetics, Braintree, MA, USA) analyses were undertaken by following the method of Suleria et al. [9].

Statistical analyses

All statistical analyses were conducted using a one-way ANOVA with Tukey's multiple and Dunnett's comparison tests. These calculations were carried out using GraphPad Prism 5 Software for Windows. Significance was observed at $P < 0.05$. IC50 values were calculated from the percentage inhibition using GraphPad Prism 5 (using variable slope response curves (four parameters)) and expressed as IC50 (with all R^2 above 0.96) with a 95% confidence interval.

Fig. 1 A schematic illustrating the chromatography strategy and pooling of fractions following AEC
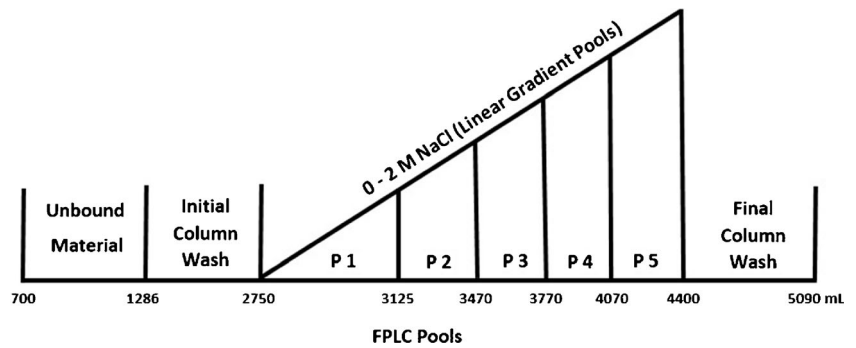
Results and discussion

Preparation and anti-thrombotic activity of hydrolysate

Research into the isolation, purification and characterisation of sulphated polysaccharides from different marine sources has increased in the last few decades, particularly in the application of these molecules as therapeutics [16]. Marine processing waste, often discarded due to limited know-how, market constraints and technological barriers, is a good source of bioactive molecules that could be extracted [17]. One of the most important research areas in this field is the isolation and purification of sulphated polysaccharides and their derivatives for the development of therapeutic products, in particular, better anti-coagulant agents with minimal side effects [18].

Marine processing waste can be hydrolysed in different ways for the release of sulphated polysaccharides. As in most of methodologies involving the isolation of sulphated polysaccharides, these techniques require the action of proteolytic enzymes followed by chromatographic separation [19]. AEC is commonly used to purify proteins, amino acids, polysaccharides and other acidic substances with a negative charge while SEC is used to obtain a refined molar mass distribution of these moieties. Therefore, it is important to develop methods to separate these molecules both on the basis of molecular size and charge distribution.

In this study, a hydrolysate was prepared from blacklip abalone viscera using a mixture of papain and bromelain enzymes. Protein and sulphated polysaccharide contents were estimated in the hydrolysate to be 12.1 ± 1.1 and 1.9 ± 0.7 mg/g of starting abalone material, respectively (on a wet weight basis). Initial screening of anti-thrombotic activity from 100 μg/mL hydrolysate (on a sulphated polysaccharide basis) via HCII-mediated thrombin inhibition was estimated. The hydrolysate inhibited thrombin by $92.5 \pm 1.4\%$ confirming blacklip abalone viscera as a source of anti-thrombotic molecules. An assessment of the enzyme-only control showed no sulphated polysaccharide content and no anti-thrombotic activity (data not shown) ensuring that all anti-thrombotic and anti-coagulant activity observed from

the abalone hydrolysate was due to abalone molecules and not the enzymes used to produce the hydrolysate. The
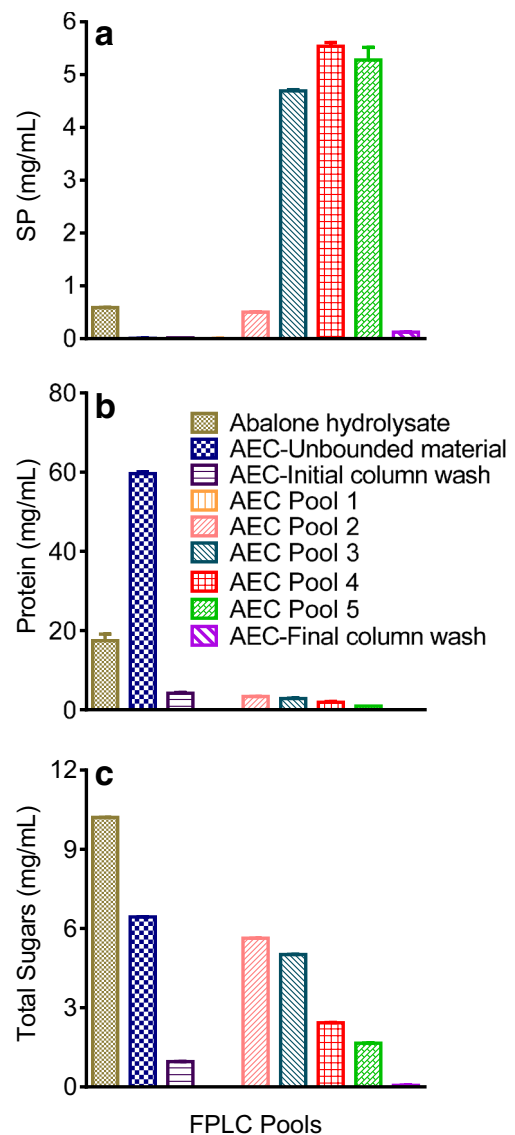

Fig. 2 Biochemical assessment of AEC pools obtained from anion exchange of the blacklip abalone hydrolysate. (**A**) Sulphated polysaccharide concentration, (**B**) total protein concentration and (**C**) total neutral sugar concentration

hydrolysate was then desalted and fractionated using anion exchange chromatography.

Preparation of anionic fractions with anti-thrombotic and anti-coagulant activity

Anion exchange chromatography

Sulphated polysaccharides with anti-coagulant activity are present in various marine invertebrates [20] and are potent inhibitors of thrombin and factor Xa mediated through antithrombin or HCII. In general, these molecules contain a high negative charge density due to the presence of sulphate and carboxyl groups that facilitates anionic separation into fractions with enhanced anti-thrombotic activity [5]. To enrich the anti-thrombotic activity in the abalone visceral hydrolysate, 800–900 mg of hydrolysate (on sulphated polysaccharide basis) was subjected to AEC. Figure 1 shows a schematic illustrating the pooling strategy of the salt eluted fractions. Fractions were pooled based on their sulphated polysaccharide concentration. Five AEC pools were prepared and termed AEC pools 1–5 throughout this study. AEC pools 1–3 were pools of fractions collected over 0.2–1.2 M NaCl, and AEC pools 4 and 5 consisted of fractions collected over 1.2–2.0 M NaCl (Fig. 1). The unbound material, initial column wash and final column wash were reserved for further analyses also.

Estimation of total protein, neutral sugars and sulphated polysaccharides

Total protein, sulphated polysaccharides and total neutral sugar concentrations were estimated in all AEC pools and are shown in Fig. 2. Most of the protein and neutral sugar content of the hydrolysate was not retained by the AEC column. The sulphated polysaccharide content was concentrated to pools 3–5. The highest sulphated polysaccharide to protein ratios, 2.9 and 5.7, occurred in AEC pools 4 and 5, respectively. This ratio was 0.03 in the abalone hydrolysate, showing that AEC successfully concentrated the anionic, sulphated polysaccharides into three pools (AEC pools 3–5). The column-bound neutral sugars eluted predominantly in AEC pools 2 and 3.

Anti-thrombotic activity measured through HCII-mediated thrombin inhibition

After confirmation of sulphated polysaccharides in AEC pools, anti-thrombotic activity was measured via HCII-mediated thrombin inhibition. Table 1 shows that there was low or no anti-thrombotic activity in the unbound material, initial column wash and AEC pools 1–4. The initial hydrolysate, AEC pool 5 and the final column wash produced significant anti-thrombotic activity and showed consistent inhibition of thrombin even at lower concentrations of sulphated

Table 1 Heparin cofactor II (HCII)-mediated thrombin inhibition by abalone hydrolysate and anion exchange chromatography (AEC) pools

Sample description	Assay concentrations of samples (µg/mL SP)					IC50 (µg/mL SP)
	100	50	10	5	1	
Abalone hydrolysate	92.5 ± 1.4b	83.7 ± 1.1b	26.3 ± 1.6c	5.8 ± 0.7c	0	16.4 (14.3–18.3)
Unbound material	0	0	0	0	0	–
Initial column wash	0	0	0	0	0	–
AEC pool 1	0	0	0	0	0	–
AEC pool 2	0	0	0	0	0	–
AEC pool 3	0	0	0	0	0	–
AEC pool 4	12.2 ± 1.8c	10.1 ± 1.9c	9.1 ± 1.1d	0	0	–
AEC pool 5	96.4 ± 0.7a	93.6 ± 0.7a	76.4 ± 2.3a	38.4 ± 1.4a	0	5.7 (5.4–6.0)
Final column wash	93.4 ± 0.8b	91 ± 0.6a	58.1 ± 1.3b	35.3 ± 0.7b	8.4 ± 1.5	7.8 (7.2–8.5)
Standard	16	8	4	2	1	–
Heparin	91.5 ± 0.6	87.6 ± 1.1	75.0 ± 1.3	48.0 ± 2.1	30.9 ± 1.7	2.1 ± 0.5 (2.2–2.9)

Percentage inhibition of thrombin mediated through HCII at 10 min

Letters show significant differences ($P < 0.05$) between samples at each concentration based on a one-way ANOVA, with Tukey's multiple comparison test

SP sulphated polysaccharides, *IC50* inhibitor concentration at 50% expressed as mean (with 95% confidence interval)

polysaccharides (1–10 µg/mL). Aside from standard heparin, the highest inhibition of thrombin was observed in response to AEC pool 5 showing that AEC had concentrated the anti-thrombotic activity. Also the IC50 values in Table 1 show that AEC improved anti-thrombotic activity as evidenced by a decrease in the IC50 value by almost threefold for AEC pool 5 (5.8 µg/mL) relative to that of the hydrolysate (16.5 µg/mL).

Anti-coagulant activity in blood and plasma

The anti-coagulant effect of the abalone hydrolysate and AEC pools and washes was measured in plasma using PT and aPTT assays and in whole blood using TEG assays. The PT assays showed that the unbound material, initial column wash, AEC pools 1–3 and the final column wash did not prolong PT (data not shown) above the negative control. Table 2 shows PT was significantly prolonged in response to the abalone hydrolysate and AEC pools 4 and 5 and also shows that results from the aPTT assays were similar, revealing significant increases in aPTT on addition of the hydrolysate, AEC pools 4 and 5. Unlike the PT results, the final column wash showed good activity. Overall, AEC pools 4 and 5 prolonged both PT and aPTT more effectively than the original extract indicating that the AEC had concentrated the anti-thrombotic activity into these pools.

The abalone hydrolysate, AEC pools 4 and 5 and the final column wash were further assessed for anti-coagulant activity by TEG assay with no anti-coagulant effect observed in response to the column wash in this assay (data not shown). In Table 3, *R* time was prolonged significantly by the hydrolysate and AEC pools 4 and 5 compared to the saline control. Interestingly, AEC pool 4 showed higher activity than AEC pool 5 as evidenced by the similar *R* times achieved by 25.6 µg/mL AEC pool 4 and 366.6 µg/mL AEC pool 5. Furthermore, no clotting was observed in response to 38.5 µg/mL AEC pool 4. With respect to, furthermore, AEC pool 4 reduced the α-angle and MA value more effectively than the hydrolysate and AEC pool 5 (Table 3).

Preparation of size exclusion fractions (from AEC pools) with anti-thrombotic and anti-coagulant activity

Separation of AEC pools using SEC-FPLC

The AEC pools 4 and 5 which demonstrated consistent and significant anti-thrombotic and anti-coagulant activity were further resolved by SEC. Depending upon the source, sulphated polysaccharides from marine organisms vary in chemical composition, structure and molecular weight. In this study, we used size exclusion chromatography to further separate sulphated polysaccharides from a blacklip abalone (*H. rubra*) hydrolysate into fractions with enhanced anti-thrombotic and anti-coagulant activity. Sulphated

Table 2 Prothrombin time (PT) and activated partial thrombin time (aPTT) of anion exchange chromatography (AEC) pools

Sample descriptions	SP (µg/mL)	Time (s)	INR
PT			
Saline control	0	10.7 ± 0.03	1.0
Abalone hydrolysate	59.0	10.9 ± 0.05	1.01
	59.9	$11.6 \pm 0.06^{*}$	1.08
	70.8	$12.5 \pm 0.02^{*}$	1.1
	94.4	$15.4 \pm 0.04^{**}$	1.4
	118.1	$16.4 \pm 0.05^{**}$	1.5
AEC pool 4	55.4	11.0 ± 0.05	1.02
	73.8	11.5 ± 0.06	1.07
	92.3	$13.0 \pm 0.02^{*}$	1.2
	110.8	$15.8 \pm 0.04^{**}$	1.4
	138.5	$18.9 \pm 0.05^{**}$	1.7
AEC pool 5	52.7	11.8 ± 0.05	1.1
	65.9	$12.9 \pm 0.06^{*}$	1.2
	87.9	$19.3 \pm 0.02^{**}$	1.8
	105.5	$21.2 \pm 0.04^{**}$	1.9
	131.9	$29.0 \pm 0.05^{***}$	2.7
aPTT			
Saline control	0	32.4 ± 0.03	1.0
Abalone hydrolysate	3.0	$37.9 \pm 0.05^{*}$	1.2
	3.9	$39.3 \pm 0.02^{*}$	1.3
	7.4	$52.8 \pm 0.05^{**}$	1.8
	14.8	$76.1 \pm 0.03^{***}$	2.8
AEC pool 4	3.5	$40.3 \pm 0.05^{*}$	1.3
	4.3	$46.6 \pm 0.04^{*}$	1.5
	5.0	$57.7 \pm 0.05^{**}$	2.0
	5.5	$73.2 \pm 0.12^{***}$	2.7
AEC pool 5	5.9	$40.9 \pm 0.05^{*}$	1.3
	8.8	$49.6 \pm 0.06^{**}$	1.9
	10.6	$55.4 \pm 0.02^{**}$	1.9
	21.1	$90.5 \pm 0.05^{***}$	3.4
AEC column wash	3.1	38.9 ± 0.05	1.2
	4.1	$42.1 \pm 0.06^{*}$	1.4
	6.2	$46.8 \pm 0.02^{*}$	1.6
	12.4	$80.7 \pm 0.05^{***}$	3.0

Statistical significance was determined using a one-way ANOVA with Dunnett's multiple comparison test compared to saline control with $P < 0.05$, $P < 0.01$ and $P < 0.001$

SP sulphated polysaccharides, *INR* international normalised ratio

$^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$

polysaccharides with similar anti-thrombotic activity have been previously investigated in other abalone species, like *H. discus hannai* Ino, *Haliotis diversicolor* Reeve and *Haliotis discus discus* Reeve, and effectively separated into pools of more bioactive molecules using ion exchange and size exclusion chromatography [5].

Table 3 Thromboelastography of abalone hydrolysate and anion exchange chromatography (AEC) pools

Sample descriptions	SP (μg/mL)	R time (min)	Angle (°)	MA (mm)
Saline control	0	11.8 ± 0.8	32.9 ± 2.5	48.2 ± 0.4
Abalone hydrolysate	19.7	12.5 ± 0.3	29.3 ± 0.8***	46.2 ± 0.6
	25.3	13.8 ± 0.3	21.9 ± 0.6***	38.9 ± 0.2***
	35.8	22.1 ± 0.2***	18 ± 0.8***	31.4 ± 0.6***
	54.4	31.2 ± 0.7***	14.9 ± 0.2***	24.5 ± 0.8***
AEC pool 4	15.4	12.5 ± 0.1	26.7 ± 1.5***	45.7 ± 1.8
	19.2	15.3 ± 0.5*	18.8 ± 0.8***	37.6 ± 0.4***
	25.6	20.1 ± 0.3***	9.7 ± 0.2***	30.5 ± 2.5***
	38.5	No clot formed[a]		
AEC pool 5	73.3	10.7 ± 0.5	36.2 ± 1.3*	46.7 ± 2.2
	146.6	10.9 ± 0.2	37.3 ± 0.7**	48.6 ± 0.6
	183.3	11.6 ± 0.3	29.6 ± 1.9***	45.3 ± 1.7
	244.4	14 ± 0.8	18.4 ± 2.3***	34.2 ± 2.6***
	366.6	18.8 ± 0.2***	7.6 ± 0.1***	17 ± 1.5***

Statistical significance was determined using a one-way ANOVA with Dunnett's multiple comparison test compared to saline control with $P < 0.05$, $P < 0.01$ and $P < 0.001$

SP sulphated polysaccharides, R reaction time until clot formation, MA maximum amplitude of formed clot, MA maximum amplitude of formed clot

*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

[a] No clot formed in response to, or greater than, 38.5 μg/mL sulphated polysaccharide of SEC pool A of AEC pool 4

To separate AEC pools 4 and 5 on the basis of molecular size, SEC was performed using 0.2 M NaCl to minimise interactions between the abalone molecules and the resin. All SEC fractions were collected and analysed for the presence of sulphated polysaccharides and protein to provide the elution profiles presented in Fig. 3. On this basis, the SEC fractions were pooled. AEC pool 4 generated three SEC pools termed SEC pools A, B and C). AEC pool 5 generated four pools known as SEC pools A, A*, B and C. All SEC pools were desalted using 3-kDa spin columns before total protein, neutral sugars and sulphated polysaccharide contents were estimated.
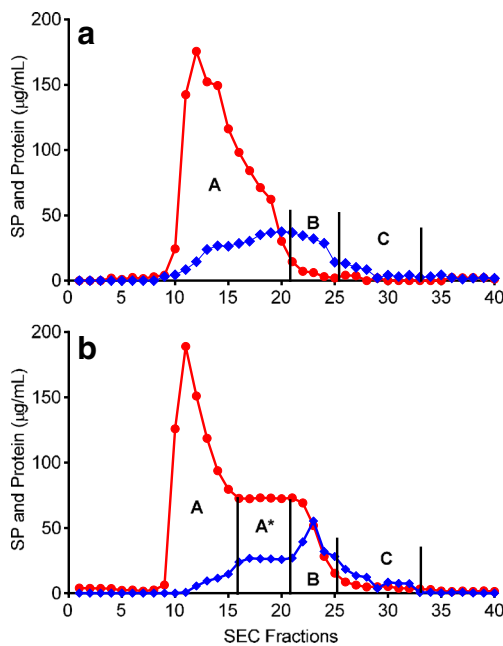

Fig. 3 SEC-FPLC elution profiles of AEC pools 4 and 5. (**A**) AEC pool 4 and (**B**) AEC pool 5. During the SEC, 40 sequential fractions were collected and subjected to DMMB assay to estimate the sulphated polysaccharides (SP) (■) and BCA assay to estimate protein content (■) in micrograms per millilitre. Based on these data, fractions were pools A–C were prepared
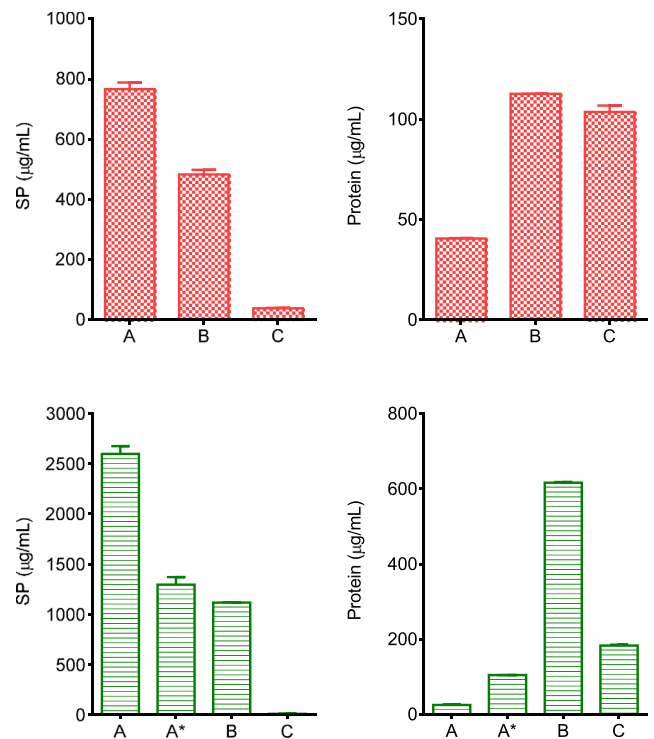

Fig. 4 Protein and sulphated polysaccharide concentration in SEC pools derived from AEC pools 4 and 5. (**A**) Concentration of sulphated polysaccharides and (**B**) total protein in SEC pools SEC pools derived from AEC pool 4 (■); (**C**) concentration of sulphated polysaccharides and (**D**) total protein in SEC pools derived from AEC pool 5 (■)

Estimation of total protein and sulphated polysaccharides

The total protein and sulphated polysaccharide contents (using Blyscan) were estimated in all SEC pools (Fig. 4). It was observed that the SEC pools A and A* were enriched in sulphated polysaccharides but had low protein content thus higher sulphated polysaccharide to protein ratio as compared to SEC pools B and C. For example, SEC pool A from AEC pool 5 had a sulphated polysaccharide-to-protein ratio of approximately 103 as compared to that of SEC pool A of AEC pool 4 at approximately 19. Following this confirmation of further separation of the sulphated polysaccharides from protein present in the abalone hydrolysate, anti-thrombotic activity was assessed via HCII-mediated thrombin inhibition.

Anti-thrombotic activity measured through HCII-mediated thrombin inhibition

In vitro HCII-mediated anti-thrombotic activity, expressed as percentage inhibition of thrombin at 10 min, is presented in Table 4 and shows that SEC pool A* from AEC pool 5 produced the highest thrombin inhibition across all concentrations and was comparable to standard heparin. The IC50 value of 1.8 µg/mL for SEC pool A* from AEC pool 5 is comparable to the IC50 calculated for standard heparin (2.1 µg/mL). These results confirmed that the SEC further enriched sulphated polysaccharides with anti-thrombotic activity. Assessment of anti-coagulant activity was then performed using PT and aPTT assays in plasma and TEG assays in whole blood.

Anti-coagulant activity in blood and plasma

All SEC pools from AEC pools 4 and 5 were assayed in plasma with no anti-clotting activity observed in response to SEC pools B and C (data not shown). Table 5 shows anti-thrombotic activity in response to SEC pool A derived from pool 4 with SEC pools A and A* from AEC pool 5. These fractions showed increased PT and produced higher INRs when compared to other SEC pools at similar or higher sulphated polysaccharide concentrations. The same trends were observed for aPTT; however, SEC pool A* from AEC pool 5 extended aPTT similarly compared to SEC pool A from AEC pool 4. In whole blood, all SEC pools A and A* had significant anti-coagulant activity.

The TEG data in Table 6 shows that SEC pool A from AEC pool 4 had higher activity than other SEC pools given that there was no blood coagulation in response to 20.5 µg/mL, whereas clots were still being formed in response to SEC pools A and A* from AEC pool 5 at 108.2 and 35.9 µg/mL, respectively. Higher anti-coagulant activity also appeared to be associated with stronger effects on α-angle and MA values as they decreased significantly compared to the saline control. These results suggest that molecules present in SEC pools A and A* affect clot strength and platelet function indicating that the kinetics of fibrin polymerisation and networking may be affected.

Different anti-coagulant responses were observed in response to different SEC pools also. For example, SEC pools A and A* from AEC pool 5 generally prolonged aPTT and PT more than SEC pool A from AEC pool 4; however, in whole

Table 4 Heparin cofactor II (HCII)-mediated thrombin inhibition of size exclusion chromatography (SEC) pools collected from anion exchange chromatography (AEC) pools

Percentage inhibition of thrombin mediated by HCII at 10 min						
Sample description	Assay concentrations of samples (µg/mL SP)					IC50 (µg/mL)
	50	10	5	1	0.5	
AEC pool 4						
SEC pool A	25.8 ± 2.4f	18.2 ± 1.7d	7.6 ± 1.2e	3.9 ± 2.6c	0	7.6 (5.6–10.3)
SEC pool B	0	0	0	0	0	–
SEC pool C	0	0	0	0	0	–
AEC pool 5						
SEC pool A	85.8 ± 1.4c	50.7 ± 0.3c	27.7 ± 2.6c	0	0	8.3 (7.3–9.4)
SEC pool A*	96.3 ± 0.4a	93.1 ± 1.1a	89.1 ± 0.6a	40.1 ± 3.3a	29.7 ± 0.8	1.8 (1.4–2.2)
SEC pool B	92.7 ± 0.8b	75.9 ± 2.3b	45.4 ± 3.5b	0	0	5.0 (4.6–5.5)
SEC pool C	0	0	0	0	0	–
Heparin	8	4	2	1	0.5	–
	89.0 ± 2.5	72.3 ± 1.8	66.3 ± 2.4	38.8 ± 1.1	29.9 ± 1.8	2.1 ± 0.5 (0.8–3.0)

Letters show significant differences ($P < 0.05$) between samples at each concentration based on a one-way ANOVA, with Tukey's multiple comparison test

SP sulphated polysaccharides, *IC50* inhibitor concentration at 50% expressed as mean with 95% confidence interval span

Table 5 Prothrombin time (PT) and activated partial thrombin time (aPTT) of size exclusion chromatography (SEC) pools collected from anion exchange chromatography (AEC) pools

Sample descriptions	SP (µg/mL)	Time (s)	INR ratio
PT			
Saline control	0	14.7 ± 0.01	1.0
AEC pool 4 (SEC pool A)	38.3	18.4 ± 0.2*	1.3
	51.1	23.8 ± 0.1**	1.6
	76.6	28.8 ± 0.3***	2.0
AEC pool 5 (SEC pool A)	34.6	20.1 ± 0.1*	1.4
	51.9	27.9 ± 0.1**	1.9
	64.9	31.2 ± 0.2***	2.1
AEC pool 5 (SEC pool A*)	17.2	24.2 ± 0.1**	1.7
	25.8	29.9 ± 0.1***	2.0
	32.3	36.2 ± 0.2***	2.5
aPTT			
Saline control	0	39.5 ± 0.3	1.0
AEC pool 4 (SEC pool A)	2.6	55.3 ± 0.4**	1.9
	3.1	60.7 ± 0.3**	2.1
	3.8	90.4 ± 0.3***	3.4
AEC pool 5 (SEC pool A)	6.5	46.5 ± 0.9*	1.5
	7.4	56.5 ± 0.7**	1.9
	8.7	68.3 ± 0.5**	2.4
AEC pool 5 (SEC pool A*)	3.2	61.5 ± 0.7**	2.2
	3.7	77.5 ± 0.7***	2.8
	4.3	98.1 ± 0.6***	3.8

Statistical significance determined using a one-way ANOVA with Dunnett's multiple comparison test compared to saline control with $P < 0.05$, $P < 0.01$ and $P < 0.001$

SP sulphated polysaccharides, IC50 inhibitor concentration at 50% expressed as mean with 95% confidence interval span

*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

blood, SEC pool A from AEC pool 4 displayed the highest anti-coagulant response preventing clot formation at concentrations two- to fivefold less than SEC pool A and A* (respectively) from AEC pool 5. It is reported that an increase in aPTT reflects involvement in the intrinsic coagulation pathway, while an increase in PT reflects the extrinsic coagulation pathway. Furthermore, prolongation in aPTT generally reflects abnormality or deficiency of factors VIII, IX, XI, XII, X and II and high molecular weight kininogen, prekallikrein and fibrinogen, whereas PT is sensitive to coagulation factors V, VII and X [21, 22]. It is possible that one of more different sulphated polysaccharides or negatively charged polysaccharides are present in blacklip abalone that are responsible for anti-thrombotic activity.

Overall, both PT and aPTT were significantly prolonged more by SEC pools as compared to AEC pools. For example in the PT assay, AEC pool 5 achieved an INR of 1.1 from 52.7 µg/mL, whereas SEC pools A and A*, collected from AEC pool 5, achieved an INR of 1.9 and 2.0 from 51.9 and 25.9 µg/mL, respectively, effectively prolonging PT by two- to fourfold. This was also observed in whole blood were AEC pool 4 formed no blood clot in response to ≥38.5 µg/mL (sulphated polysaccharides), whereas SEC pool A from AEC pool 4 prevented clot formation at a lower concentration (≥20.5 µg/mL) indicating that SEC improved anti-coagulant activity twofold.

Increased anti-coagulant activity also appeared to be associated with greater effects on α-angle and MA values as they decreased significantly compared to the saline control. These

Table 6 SEC exclusion pools and thromboelastography

Sample descriptions	SP (µg/mL)	R (min)	Angle (°)	MA (mm)
Saline control	0	13.3 ± 1.1	27.9 ± 2.1	46.9 ± 1.7
AEC pool 4 (SEC pool A)	5.3	16.6 ± 1.6	17.8 ± 1.2***	26 ± 2.1***
	6.4	22.7 ± 2.1***	16.8 ± 2.8***	24.4 ± 1.8***
	10.6	26.8 ± 1.4***	11 ± 1.3***	25.9 ± 3.1***
	12.8	33.2 ± 2.3***	7.2 ± 1.9***	24.1 ± 2.9***
	20.5	No clot formed[a]		
AEC pool 5 (SEC pool A)	36.1	13.6 ± 1.9	29.4 ± 2.3	42.5 ± 1.9
	54.1	18.7 ± 1.4**	10 ± 1.2***	38 ± 2.3***
	72.1	19.4 ± 2.1**	5.9 ± 1.9***	8.4 ± 1.7***
	108.2	22.8 ± 1.1***	4.2 ± 2.3***	7.1 ± 1.4***
AEC pool 5 (SEC pool A*)	18.0	11.7 ± 2.3	33.9 ± 3.2***	31.6 ± 2.5***
	20.2	13.2 ± 1.1	26 ± 1.1	31.2 ± 2.4***
	27.0	18 ± 2.9**	11.2 ± 2.6***	18.9 ± 1.7***
	35.9	22.3 ± 2.3***	8.4 ± 1.2***	9.8 ± 1.9***

Statistical significance determined using a one-way ANOVA with Dunnett's multiple comparison test compared to saline control with $P < 0.05$, $P < 0.01$ and $P < 0.001$

SP sulphated polysaccharides, R reaction time until clot formation, MA maximum amplitude of formed clot

*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

[a] No clot formed indicated no blood clot formation in response to, or greater than, 38.5 µg/mL sulphated polysaccharide of SEC pool A of AEC pool 4

results suggest that sulphated polysaccharide present in blacklip abalone extracts affects clot strength and platelet function indicating that the kinetics of fibrin polymerisation and networking may also be affected. Current research regarding the blacklip abalone extracts provides evidence that their anti-thrombotic and anti-coagulant activity is due to the presence of sulphated polysaccharides; further investigation into molecular structure and composition is required.

Conclusion

In this study, a new source of anti-thrombotic and anti-coagulant molecules was found in blacklip abalone viscera. Separation of a hydrolysate using AEC and SEC produced fractions enriched with sulphated polysaccharides and enhanced with anti-thrombotic and anti-coagulant activity. Although the recovery of sulphated polysaccharides from blacklip abalone viscera was low (approximately 0.2% on a wet weight basis), anti-thrombotic activity was comparable to commercial heparin and may therefore only require low inclusion in a nutraceutical product.

These findings indicate the potential value of this waste source in providing ingredients for nutraceuticals and functional foods targeted to preventing unwanted clot formation leading to deep vein thrombosis or pulmonary embolism in people at risk. For example, this type of product may be taken prophylactically prior to long distance air travel to help prevent economy class syndrome or during post-operative care. Further research is required into the efficacy of these molecules in vivo.

Author contributions Hafiz Ansar Rasul Suleria, Simone A. Osborne and Paul Masci conceived and designed the experiments. Hafiz Ansar Rasul Suleria performed all experiments. Rama Addepalli and Wei Chen provided training and assistance with 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.

Compliance with ethical standards

Conflicts of interest   The authors have no conflict of interest.

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