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Extraction, structural characterization and stability of polyhydroxylated naphthoquinones from shell and spine of New Zealand sea urchin (*Evechinus chloroticus*)

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ABSTRACT

The extraction of polyhydroxylated naphthoquinone (PHNQ) pigments from the shell and spines of the New Zealand sea urchin *Evechinus chloroticus* was evaluated using six different macroporous resins as an alternative to using organic solvent extraction alone. Four of the resins evaluated in this study (D4006, D4020, D101 and NKA-9) provided the best extraction of PHNQ pigments in terms of the overall adsorption and desorption of *E. chloroticus* PHNQ pigments from the resins. Organic solvents alone had a higher yield of PHNQs than the resins. The PHNQ composition was characterised by high-performance liquid chromatography (HPLC) with diode-array detection and mass spectrometry. Five PHNQ compounds (spinochromes E, B, C, A and echinochrome A), and three aminated PHNQ compounds (spinamine E, echinamines A and B) were identified. The pigments were found to be prone to degradation on exposure to light, with the aminated PHNQ pigments being the least stable.

1. Introduction

Evechinus chloroticus (also known as kina) is a sea urchin species that is indigenous to New Zealand. *E. chloroticus* has been traditionally sought after by people of the Pacific region for its edible roe and has been harvested commercially (Barker, 2013). The harvesting of sea urchin roe results in the generation of a considerable amount of shell and spine as a waste product that can contribute to environmental issues including generation of bad odours, contamination of air, soil pollution, and damage to the marine ecosystem (Achilias, 2012). Investigation of possible ways to utilize the shell and spine waste may add value and potentially resolve issues associated with environmental pollution.

Echinochrome A, a polyhydroxylated naphthoquinone (PHNQ) pigment, was first isolated by MacMunn in 1885 from the perivisceral (coelomic) fluid of *Echinus esculentus* (Tyler, 1939). Since then, more than 30 PHNQ pigments have been discovered and identified as spinochromes from different species of sea urchins (Shikov, Pozharitskaya, Krishtopina, & Makarov, 2018; Vasileva, Mishchenko, & Fedoreyev, 2017; Zhou et al., 2011). PHNQ are polyhydroxylated derivatives of either juglone (5-hydroxy-1,4-naphthoquinone) or naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) that are usually substituted with ethyl, acetyl, methoxyl, and amino groups (Anderson, Mathieson, & Thomson, 1969). PHNQs are excellent scavengers of free radicals through donating hydrogen atoms in a similar way to other well-known polyphenolic antioxidants such as the catechins, quercetin, and gallic acid (Powell, Hughes, Kelly, Conner, & McDougall, 2014). Furthermore, PHNQs have been shown to exhibit antimicrobial activity against both model human pathogenic bacteria and marine strains (Brasseur et al., 2017). Also, PHNQs have been demonstrated to exhibit protective effects against several human health disorders such as cardiovascular diseases, eye conditions, and anti-inflammation activity (Egorov et al., 1998; Brasseur et al., 2017). These properties have formed the basis for their potential application not only as natural food colourants (Zhou et al., 2011), but increasingly also as functional ingredients in foods and nutraceutical products.

PHNQ pigments occur in the shell and spines of the sea urchin as pigment granules associated with Ca^{2+} and Mg^{2+} in the mineral structure, and the protein matrix. Hence, acidic solutions have been used to dissolve the mineral structure of the sea urchin shell and spine,

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followed by extraction of the pigments from the acid solution with organic solvents, and then further purification using chromatography techniques. Diethyl ether has been used in several studies to extract PHNQ pigments after dissolving the mineral structure using HCl (Kuwahara et al., 2009; Kuwahara, Hatate, Chikami, Murata, & Kijidani, 2010; Shikov et al., 2011). Other organic solvents such as chloroform (Vasileva et al., 2017), ethyl acetate (Powell et al., 2014), either individually or in combination (Vasileva et al., 2017), have also been used for the extraction of PHNQs. Zhou and co-workers (Li et al., 2013; Zhou et al., 2011) evaluated the extraction of PHNQs from *Strongylocentrotus nudus*, *Glyptocidaris crenularis* and *Strongylocentrotus intermedius* using macroporous resin as an alternative to the use of traditional extraction of PHNQs with organic solvents that may expose operators and the environment to toxic compounds. Hence the use of macroporous resins has been proposed as a more environmental and safer option for the extraction and purification of PHNQs.

Currently, more than 1000 sea urchin species are known (Kroh & Mooi, 2018), but only a very limited number of investigations have been performed on PHNQs in these species to date. A considerable variation in PHNQ composition has been reported among different species, and even in the same species found in different geographical locations (Vasileva et al., 2017). *E. chloroticus*, unlike other species of sea urchins distributed in the Northern Hemisphere, is only found around the main islands of New Zealand and the PHNQ composition has not been reported. *E. chloroticus* has been exploited by commercial fisheries and has potential for export to countries such as Japan where the gonads, known as uni, are considered as delicacies and are highly prized.

The aim of the present study was to evaluate the extraction of PHNQs from *E. chloroticus* shell and spine using six different macroporous resins in comparison to extraction with organic solvents alone. In addition, the extracted PHNQs were fractionated by HPLC, characterised by mass spectrometry and examined for their stability to exposure to light.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical grade and unless otherwise stated, were from Fisher Scientific UK (Loughborough, Leics, UK). Hydrochloric acid, sodium hydroxide, anhydrous sodium sulfate, diethyl ether and ethyl acetate were from ECP Ltd, Auckland, New Zealand. Solvents used in high performance liquid chromatography (HPLC), formic acid, methanol and acetonitrile were of liquid chromatography grade and were from Fisher Scientific UK (Loughborough, Leics, UK. Milli-Q water was obtained from a Millipore water purification system (Millipore, USA) and used in all experiments. Macroporous resins (NKA-9, NAK-II, ADS-17, D4020, D4006, and D101) were obtained from Cangzhou Bon Absorber Technology Co., Ltd (Cangzhou, China).

2.2. Source of sea urchin shells and spines

Sea urchins (*E. chloroticus*) were collected from around the southern coast of the South Island of New Zealand by a commercial company (Cando Fishing Ltd., Bluff, New Zealand). Sea urchin shells with spines were couriered to the University of Otago the same day. The shells with spines were washed with cold water, air-dried in a fume cupboard in the dark and then the shell and spines were separated by hand and separately ground into a powder. The powder was sieved (mesh size 450 μm) and stored at −20 °C in the dark.

2.3. Extraction of PHNQ pigments using macroporous resins

The dried shell and spine powders were dissolved by gradually adding 3 M HCl to achieve a solid to liquid ratio of 1:10 (w/v) with continuous stirring as described by Zhou et al. (2011). After centrifugation at 13,300 *g* for 20 min at 4 °C, the supernatant containing the PHNQ pigments was collected and stored at −20 °C in the dark.

Six different types of macroporous resins (polar resins NKA-9 and NKA-II; intermediate polar resin ADS-17; non-polar resins D4020, D4006, and D101) were used in this study. The resins were equilibrated in 95% ethanol for 24 h and then washed thoroughly with Milli-Q water several times until the water was clear. The resins were then treated with two bed volumes of 5% (v/v) HCl and then two bed volumes of 5% (v/v) NaOH to remove any monomeric and porogenic agents that had remained in the pores of the resin after the resin synthesis process. The resins were then washed with Milli-Q water until the pH of the wash water was neutral, and then soaked in two bed volumes of 95% (v/v) ethanol for 24 h and washed thoroughly with Milli-Q water (Zhang, Jiao, Liu, Wu, & Zhang, 2008).

The pH of the crude PHNQ extract solution prepared from *E. chloroticus* shell and spine powder was adjusted to pH 1.0 using HCl. After centrifugation (13,300 *g*, 20 min, 4 °C), aliquots of the supernatant were used to evaluate binding of the pigments to each of the macroporous resins. Aliquots (5 ml) of pigment solution were mixed with 1.0 g of each resin in a 50-ml beaker, which was shaken at a constant speed of 150 rpm for 3 h. Then, the solution containing any unbound pigment was removed and retained, and the resin containing absorbed pigment was washed with Milli-Q water. The bound pigment was desorbed with 5 ml methanol in a 50-ml beaker with shaking at 150 rpm at room temperature for 1 h.

The adsorption capacity and desorption capacity of pigment were calculated using the following equations, as used by Zhou et al. (2011):

$$\text{Pigment adsorption capacity}\,(\%) = (A_1 - A_2)/A_1 \times 100\%$$

$$\text{Pigment desorption capacity}\,(\%) = A_3/(A_1 - A_2) \times 100\%$$

where A_1 is the absorbance of the pigment solution before adsorption, A_2 is the absorbance of the pigment solution after adsorption, A_3 is the absorbance of the pigment solution after desorption. The absorbance was measured at 475 nm.

2.4. Extraction of PHNQ pigments using organic solvents

The dried shell and spine powders were dissolved by gradually adding 6 M HCl at a solid to liquid ratio of 1:5 (w/v) in the dark at room temperature according to a published study (Shikov et al., 2011). The mixture was centrifuged (13,300 *g*, 20 min, 4 °C), and aliquots of the clarified supernatant containing the PHNQ pigments were extracted three times sequentially with the same volume of different organic solvents: diethyl ether, solvent treatment 1 (ST1), ethyl acetate (ST2), chloroform (ST3), and an ethyl acetate extract obtained after prior extraction with chloroform (ST4). The organic solvent extracts containing the pigments were washed with Milli-Q water to remove any residual acid and then dried over anhydrous sodium sulfate. The organic solvent was evaporated to dryness under reduced pressure in a rotary evaporator at 45 °C. The PHNQ pigments were then re-dissolved in methanol and stored at −20 °C in the dark.

2.5. Characterization of PHNQs by HPLC

HPLC analysis of PHNQs was conducted according to the method of Kuwahara et al. (2009). Prior to HPLC the PHNQ methanol solution (concentration range 5–10 mg/ml) was filtered through a 0.2 μm PTFE filter (Axiva). Aliquots of the pigment solution were injected manually using a 20 μL loop on to a reversed-phase column (C_{18} Luna 250 × 4.6 mm, 5 μM particle size, 100 Angstrom pore size, Phenomenex, USA) on an Agilent 1100 HPLC (Agilent Technologies Inc., USA). The eluted compounds were monitored over the wavelength range 250–550 nm using a diode array detector. The elution solvent system