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Purification and characterisation of aquamarine blue pigment from the shells of abalone (*Haliotis* discus hannai Ino)

Zhixing Cai [1], Jiulin Wu [1], Li Chen, Wei Guo, Jianhua Li, Jiabin Wang, Qiqing Zhang *

Institute of Biomedical and Pharmaceutical Technology and College of Chemistry and Chemical Engineering, Fuzhou University, Fuzhou 350002, PR China

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ABSTRACT

Aquamarine blue pigment (ABP) from the shells of abalone (*Haliotis* discus hannai Ino) was extracted using a precipitation adsorption method and further purified via semi-preparative HPLC. The ABP with molecular weight of 582.8 was identified as a polyenic compound by NMR. The colour value of ABP was $E^{1\%}_{1cm}612\ nm = 534.3$. ABP can dissolve in water, ethanol, methanol, acetic acid and DMSO but was scarcely soluble in chloroform, aether, acetone, petroleum ether and cyclohexane. ABP was relatively stable between 25 and 100 °C, from pH 2 to pH 12, under UV-light and indoor natural light. However, it was bleached by H_2O_2 and Na_2SO_3 and even unstable under sunlight. The stability of ABP was slightly influenced by metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+} and so on) and food addictives (sodium chloride, sugar, starch and so on). This is the first report on the characterisation of pigment obtained from *Haliotis discus hannai Ino.*

1. Introduction

Colour is a vital quality attribute of foods and plays an important role in sensory and consumer acceptance of products. Based on the origin, food colours can be classified as natural, nature identical and synthetic (Sowbhagya, Smitha, Sampathu, Krishnamurthy, & Bhattacharya, 2005). Nevertheless, synthetic colourants are frequently perceived as undesirable or harmful. There is a growing interest in the development of colourants from natural sources as additives for use in the food industry, which has been encouraged by a strong consumer demand for natural products. According to statistics, the production of the pigment is increasing at a rate of 10% every year in the international market (Orzechowski, Ostaszewski, Jank, & Berwid, 2002; Wang, Pan, Tang, & Huang, 2006; Winterhalter, 2007).

Marine animals are often brilliantly coloured and bright colouration is widespread in both sessile and non-sessile invertebrates (Bandaranayake, 2006). Mollusc shells have vivid colours and intricate patterns. Comfort (1949a, 1951) isolated a number of acid-soluble pigments from mollusc shells by chromatography and identified porphyrins as shell pigments. Using resonance raman microspectrometry, Hedegaard, Bardeau, and Chateigner (2006) identified polyenes as the pigments of mollusc shell, while they carried out using whole shells but not the pure shell pigments. However, most studies of abalone shell focus on genetics of the shell colour and the formation (Auzoux-Bordenave et al., 2010; Graham & Sarikaya, 2000; Lin & Meyers, 2005; Liu, Wu, & Zhang, 2007) and only a few explicitly identify shell pigments. Comfort (1949b) isolated the blue pigments fraction from the shells of *Haliotis cracherodii*.

Abalone (*Haliotis* discus hannai Ino) is large algivorous marine gastropods, and the most commercially important specie of gastropods in aquaculture for Asia (Chen et al., 2005; Mai, Mercer, & Donlon, 1995). The abalone is cultured extensively in China (Qi et al., 2010). Large amounts of abalone shells as aquaculture waste are produced every year. Therefore the use of abalone shells is a very important consideration.

In the present study, we isolated a pure aquamarine blue pigment from abalone shells and further investigated its characteristics. This is a new application of precipitation adsorption method in shell pigments extraction and the first report on the characterisation of pigment obtained from *Haliotis discus hannai Ino.*

2. Materials and methods

2.1. Extraction of ABP

Shells of abalone (*Haliotis* discus hannai Ino) were purchased from Yilai Abalone Products Company (Fujian Province, China). The shells were washed thoroughly with tap water and dried by forced air circulation. Thirty kilograms of shells were steeped twice with 150 l 5% (v/v) aqueous acetic acid for 24 h at room temperature. The coloured solution was filtered and adjusted the pH to 10.0

* Corresponding author. Tel./fax: +86 591 83725260.
E-mail address: zhangqiq@126.com (Q. Zhang).
[1] These authors contributed equally to this work and should be considered co-first authors.

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with NaOH aqueous solution, accompanied by a white flocculent precipitate. After standing the solution for 30 min, the precipitate absorbed with pigments was collected and redissolved in 10% (v/v) aqueous acetic acid. Finally, the crude ABP (1.5 g) was extracted with ethyl acetate.

2.2. Semi-preparative RP-HPLC

Isolation was carried out via a semi-preparative HPLC (EasySep-1010, Unimicro Technologies Co. Ltd., USA) with a reversed phase column (10 mm ID × 250 mm, 10 μm ODS) and an UV variable wavelength detector. The mobile phase consisted of 1% (v/v) aqueous acetic acid as solvent A and methanol as solvent B. Before delivering into the system, the mobile phase was degassed and filtered through a 0.45 μm PTFE filter using vacuum. With an isocratic elution (A: 45% and B: 55%) the main component (2.7 mg) was collected at the top of the peak at a flow rate of 5.0 ml/min while the detection was maintained at 254 nm.

2.3. Purity determination

2.3.1. HPLC

The analysis was investigated via HPLC (EasySep-1010, Unimicro Technologies Co. Ltd., USA) on Inertsil ODS (4.6 mm ID × 250 mm; 5 μm ODS) column using a mobile phase consisting of 0.1% (v/v) aqueous trifluoroacetic acid as solvent A and methanol as solvent B at a flow rate of 1.0 ml/min in an isocratic elution mode (A: 35% and B: 65%). The injection volume was 20 μl and the detection was performed at 254 nm using an UV variable wavelength detector.

2.3.2. TLC

The purified sample was applied to silica gel GF 254 TLC-plates (Qingdao Shenghai Chemical Co. Ltd., China) using dichloromethane/methanol (5:1, v/v; included 1% acetic acid) as mobile phase and then stained with *p*-anisaldehyde (to 135 ml of absolute ethanol was added 5 ml of concentrated sulphuric acid, 1.5 ml of glacial acetic acid and 3.7 ml of *p*-anisaldehyde).

2.4. UV–vis spectrophotometry

The ABP was dissolved in methanol and scanned from 200 to 800 nm by Varian Cary 50 ultraviolet–visible spectrophotometer (Varian, USA) in matched 1 cm quartz cuvettes against a corresponding solvent blank.

2.5. Infrared spectroscopy

FT-IR spectra on pellets of ABP mixed with KBr were recorded on a Nicolet Nexus 670 FT-IR spectrometer at a resolution of 4 cm^{-1}.

2.6. NMR spectrometry

Bruker Avance III 400 MHz NMR spectrometer (Bruker BioSpin Limited, Coventry, UK) was used to record the ^{1}H, ^{13}C and DEPT NMR spectra at room temperature in DMSO-d_6 with TMS as an internal standard.

2.7. ESI-mass spectrometry

MS measurements were performed in the full-scan mode over a mass range of *m/z* 50–1000 using ThermoFinnigan DECAX-30,000 LCQ ion trap mass spectrometer Deca XP (ThermoFinnigan, San Jose, CA, USA), equipped with an ESI source.

2.8. The pigment solubility

To study the solubility of ABP, pigment was added to water and organic solvents (chloroform, ethanol, methanol, acetone, acetic acid, aether, petroleum ether, cyclohexane and DMSO) with stirring at 25 °C for 10 min.

2.9. Stability studies

The pigment power was dissolved in 50% (v/v) aqueous ethanol for the stability studies. To study the effect of temperatures on colourant stability, the samples were bathed at different temperature (from 25 to 100 °C) for 1, 2 and 3 h. The influence of pH on colourant stability was performed at 25 °C for 1, 2 and 3 h with a mixture of 1 ml samples and 3 ml buffer solutions (from pH 2.0 to 12.0). H_2O_2 and Na_2SO_3 were respectively mixed with samples to obtain final concentrations designated (H_2O_2: 0.1%, 0.25%, 0.5%, 1%; Na_2SO_3: 1, 2.5, 5, 25, 50 μg/ml) and the mixture were stored at 25 °C for 1 h to investigate the oxidant and reducer effect on colourant stability. Light effect on colourant was performed with samples exposed to outdoor sunshine, ultraviolet-light and indoor nature light or stored at dark place for 1–10 d. The effect of metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Al^{3+}, Mg^{2+} and K^{+}) and food addictives (NaCl, sugar, starch, citric acid, sodium tartrate and glucose) on colourant stability was performed at 25 °C for 6, 12 and 24 h with a final concentration of 5 mM. And all samples were determined at 612 nm.

2.10. Determination of the colour value

The colour value of ABP was determined according to the basic procedure designed with minor adjustment (Tan et al., 2010). The absorbance was measured with 1.0 mg/100 ml density, l cm colour tube under the wavelength 612 nm. The colour value was expressed in the form of $E^{1\%}_{1\,cm}$ 612 nm of absorbance.

2.11. Statistical analysis

All experimental results were centered at using three parallel measurements of mean ± standard deviation. Analysis of variance was performed by ANOVA. Duncan's new multiple-range test was used to determine the differences of means. $P < 0.05$ was regarded as significant; $P < 0.01$ was considered very significant.

3. Results and discussion

3.1. Isolation of ABP and purity determination

ABP from the shells of abalone (*Haliotis* discus hannai Ino) was extracted with aqueous acetic acid. Flocculent precipitates were generated after the addition of NaOH to the colourant solution. The pigment was absorbed by the precipitates and extracted with ethyl acetate from the redissolved solution of the precipitates. Then the ABP was further purified by semi-preparative HPLC. The ABP gave a single and symmetrical peak on HPLC (Fig. 1) and exhibited only one spot (*Rf* = 0.64) on silica gel TLC-plate (date is not shown). These results indicated that the purified ABP was of high purity. Approximately 2.7 mg of highly purified ABP was obtained starting from 30 kg of abalone shell.

3.2. UV–vis spectroscopy

In Fig. 2a, the ultraviolet–visible absorption spectrum (200–800 nm) of the ABP exhibited strong optical absorbance in a spectral range from 210 to 250 nm, and medium strong absorbance