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Distribution of uronic acid-containing polysaccharides in 5 species of shellfishes

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ABSTRACT

Mollusks are a rich source of uronic acid-containing polysaccharides (UACPs) possessing biological and pharmacological activities. In the present study, UACP compositions of different tissues from 5 species of edible shellfishes, namely *Neverita didyma*, *Natica janthostomoides*, *Saxidomus purpurata*, *Cyclina sinensis*, and *Chlamys farreri* were systemically analyzed. Totally 10 UACPs were observed, including chondroitin sulfate, heparin, an identified mannoglucuronan, 3 unknown glycosaminoglycans (GAGs, U1, U3 and U6) with hexuronic acid-hexosamine repeating units, and 4 unknown non-GAGs (U2, U4, U5 and U7) with hexuronic acid-hexose repeating units. U4-7 showed tissue- and species-specificity while the other UACPs prevalently existed in these shellfish tissues. UACP compositions in tissues from the same shellfish species were similar for *S. purpurata* and *C. sinensis* but different for *C. farreri*, *N. didyma* and *N. janthostomoides*. And this investigation also indicates that the similarity of UACPs composition between different shellfishes is related to the genetic relationship.

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1. Introduction

Mollusks are a rich source of uronic acid-containing polysaccharides (UACPs) which show multiple biological and pharmacological activities (Li et al., 2011; Volpi & Maccari, 2005, 2009). Most of these UACPs from shellfishes have been characterized as glycosaminoglycans (GAGs), including heparin (HP)/heparan sulfate (HS) (Arumugam, Garg, Ajithkumar & Shanmugam, 2009; Nader et al., 1984; Nader et al., 1999), chondroitin sulfate (CS)/dermatan sulfate (DS) (de Sousa Junior, Nader & Dietrich, 1990; Yamada, Sugahara & Özbek, 2011) and hyaluronic acid (HA) (Volpi & Maccari, 2003), which are generally constructed through disaccharide repeats of N-acetylhexosamine and hexuronic acid. Although the presences of well-known GAGs in the shellfish species have been well documented, their tissue distribution in shellfishes has been seldom reported to our knowledge. Moreover, some non-GAG UACPs with repeated disaccharide units of a hexuronic acid linked to a hexose have also been found in shellfishes recently (Cao et al., 2015; Wang et al., 2015). However, the knowledge about this type of UACPs is limited by now. To our knowledge, little is known about the tissue- and species-specificity of UACPs, especially those non-GAG UACPs, so more efforts are needed to reveal their distribution in shellfishes.

Due to the high molecular weight of polysaccharides, disaccharide analysis after depolymerization is the most often used analytical measurement for UACPs. The current depolymerization methods via enzymatic digestion (Linhardt, 2001; Mourier, Anger, Martinez, Herman & Viskov, 2015) or deaminative cleavage by nitrous acid (Li, Ly & Linhardt, 2012) are very useful to analyze GAGs, but they could not cleave non-GAGs and unknown GAGs. By contract, acid hydrolysis could produce disaccharides in high yield from GAGs as well as non-GAG UACPs with an uronic acid in their repeated disaccharide unit due to the most resistance of the glycosidic linkage of uronic acid to acid (De & Timell, 1967). And disaccharide analysis upon 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization after acid hydrolysis showed potential to reveal complete UACP compositions in shellfishes (Cao et al., 2015). To obtain

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a more accurate profiling of UACPs it is necessary to improve the detection measurement of disaccharides. Multiple reaction monitoring (MRM) scan mode is unique to a triple quadrupole mass spectrometer, and it is capable of rapid, sensitive, and specific quantitation of analytes in highly-complex sample matrices (Kondrat, McClusky & Cooks, 1978; Kuzyk et al., 2009).

In the present study, tissue distribution of UACPs were demonstrated by using a triple quadrupole mass spectrometer with MRM mode to reveal tissue- and species-specificity of UACPs in shellfishes. 5 species of edible shellfishes which were found containing relatively complicated UACP composition in the whole bodies (Cao et al., 2015) were selected, and UACP compositions in their different tissues were profiled and compared.

2. Materials and methods

2.1. Materials

Natica janthostomoides (GNJ) and *Neverita didyma* (GND) from Gastropoda, and *Chlamys farreri* (BCF), *Saxidomus purpurata* (BSP), and *Cyclina sinensis* (BCS) from Bivalvia were obtained from Dalian, China (October 2015). Chondroitin sulfate (CS) and dermatan sulfate (DS) were purchased from Sigma–Aldrich (Saint Louis, America) and ChromaDex (California, America), respectively. Heparin (HP), hyaluronic acid (HA), papain (>3500 U/mg) and trypsin (>250 NF.U/mg) were from Sangon Biotechnology Co. (Shanghai, China). Abalone gonad sulfated polysaccharide (AGSP), a sulfated polysaccharide with backbone of →4)-β-GlcA(1 → 2)-α-Man(1→ repeating unit, was isolated and characterized in our laboratory (Wang et al., 2015).

2.2. Extraction of shellfish polysaccharides

After removing the shells, different tissues of the individual specimens were segmented and separately minced. After lyophilization, the tissue samples were mixed individually with 15 times of volume of ethanol-hexane mixture (ethanol:hexane = 1:2), then the organic solvent was removed after stirred for 1 h and standing overnight.

Each sample was digested in a mixture of 8 mL Cys-EDTA-2Na solution (0.05 mol/L), 32 mL phosphate buffer (pH 8.0, 0.05 mol/L), and 0.5% (w/w) trypsin at 37 °C for 4 h. Then 0.5% (w/w) of papain was added to the mixture for additional incubation at 65 °C for 3 h. The enzymatic digestion was terminated by boiling in 100 °C water bath for 5 min. After centrifugation at 12,300 × *g* for 20 min, the supernatant was added to 1.5 times volume of absolute ethanol and kept overnight at 4 °C, and then centrifuged at 12,300 × *g* for 15 min. Finally, the precipitate was collected and freeze-dried to obtain the crude polysaccharides.

2.3. Acid hydrolysis and 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization

Standard acid polysaccharides (HP, CS, HA, DS, and AGSP), and the polysaccharides isolated from shellfishes were hydrolyzed and derivatized according to the method described previously with modification (Cao et al., 2015). Briefly, 10 mg crude polysaccharides were dissolved with 1.0 mL 1.3 M trifluoroacetic acid (TFA) and heated at 105 °C for 3 h to produce disaccharides. After that, the liquid was blown to dry with nitrogen flow.

Then the dried acid hydrolysate was dissolved in 400 μL of ammonia, and was mixed with 400 μL of 0.3 M PMP methanolic solution. After incubation in water bath for 30 min at 70 °C, the solution was dried with nitrogen flow. Later, the dry residue was dissolved in 1.0 mL of water and then mixed with 1.0 mL of chloroform to extract the remaining PMP. The extraction process was repeated 3 times. Finally, the aqueous layer was filtered through a 0.22 μm membrane for analysis.

2.4. HPLC–MS/MS analysis

Hypersil GOLD C18 column (2.1 × 150 mm, 5 μm, Thermo scientific) was used and kept at 30 °C with a mobile phase of 20 mM ammonium acetate–acetonitrile (83:17, v/v) at a flow rate of 0.5 mL/min. Electrospray ionization (ESI) mass spectra were obtained by 4000 QTRAP (AB SCIEX) with multiple reaction monitoring (MRM) in the positive ionization mode. The turbo gas temperature was set at 600 °C and the ionspray voltage was adjusted to 5500 V. Other parameters were shown in Table 1.

The relative quantification of UACPs in the shellfish tissues was conducted according to peak areas of their PMP-disaccharides in MRM chromatograms with HP, CS, and AGSP as standards. And those unknown UACPs were quantified using the calibration curve of selected UACP standards with similar ion abundances.

3. Results and discussion

3.1. Characterization of disaccharides from uronic acid-containing polysaccharides

Disaccharide fragments released from UACPs of different shellfish tissues were analyzed by HPLC–MS/MS upon PMP derivatization. Observation of each PMP-disaccharide in the MRM chromatogram suggests the existence of corresponding UACPs. Due to the high sensitive of MRM mode and the segment of shellfish tissues, the species of UACPs found in the present study was much more than in the previous investigation with ion trap mass spectrometer. Thus, totally 10 PMP-disaccharides were detected, which corresponds to 3 identified polysaccharides (CS, HP, and AGSP), and 7 unidentified polysaccharides (U1–U7). Besides retention time

Table 1
Parameters for MRM analysis of PMP-labeled disaccharides.

No.	Q_1 Mass (Da)	Q3 Mass (Da)	Time (msec)	CE (volts)	DP (volts)	EP (volts)	CXP (volts)
1	686.30	510.12	150	40	80	10	12
2	686.30	492.10	150	40	100	10	12
3	687.30	511.20	150	40	100	10	12
4	686.30	525.20	150	30	90	10	12
5	687.30	373.00	150	50	100	10	12
6	687.30	187.00	150	70	90	10	12
7	686.30	175.00	150	70	100	10	12
8	686.30	187.00	150	70	100	10	12
9	687.30	175.00	150	70	90	10	12
10	686.30	372.00	150	50	100	10	12

CE: collision energy; DP: declustering potential; EP: entrance potential; CXP: collision cell exit potential.

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