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High-Performance Liquid Chromatographic Determination of Glycogen in Sea Urchin Gonads with Refractive Index Detection

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Abstract

A high-performance liquid chromatographic method using refractive index detection for the determination of glycogen in sea urchin *(Paracentrotus lividus)* gonads is developed. After alkaline digestion with sodium carbonate, samples are adjusted to pH 4.6 with citric acid and incubated with amyloglucosidase to hydrolyze the glycogen. The resulting glucose is determined using a Spherisorb NH_2 column as the stationary phase and an acetonitrile–water mixture (80:20, v/v) as the mobile phase. The relative standard deviation (%) was 3.57, the limit of detection was 40.1 µg/mL, and the recovery percentage was 97.2%.

Introduction

The climate and isolated nature of the Atlantic coast in Galicia (northwest Spain) with its many estuaries have proved ideal for the growth of many species of shellfish. Galicia is the top European country in the production of sea urchins *(Paracentrotus lividus)* and fourth in the world after Chile, Japan, and USA (1). The sea urchin market was originally limited to the coastal region because of the highly perishable characteristic of the product; however, the development of canning processes has allowed for the expansion of the market, and the sea urchin industry has become increasingly important to the economy in Galicia in recent years (2).

It is well-known that the glycogen content in shellfish is much higher than that of fish (3). Glycogen is a branched polymer of α-D-glucose residues linked through α(1→4)- and α(1→6)-glycoside bonds (4). Traditionally, there have been two methods for measuring glycogen, either by acid hydrolysis or by enzymatic hydrolysis (5). Chemical hydrolysis has been criticized for its lack of specificity; however, the use of specific enzymatic hydrolysis has been shown to be advantageous as an accurate and quantitative measurement of glycogen (6). Fungal amyloglucosidase hydrolyzes the α(1→4)-linkage and also the α(1→6)-glycoside bond of glycogen, thus it is well-suited for the complete degradation of glycogen to glucose (7).

Previous methods for the determination of formed glucose have used colorimetric enzyme kits based either on the hexokinase/glucose-6-phosphate dehydrogenase system (8) or glucose oxidase (9); however, to our knowledge, no chromatographic techniques have been reported. In this study, a method for the determination of glycogen in sea urchin gonads by high-performance liquid chromatography (HPLC) with refractive index (RI) detection has been developed.

Experimental

Samples

Sea urchins were caught off the Atlantic coastal region near Touriñán in Galicia (northwest Spain) in February, 1999. After the catch, the gonads (which constitute the edible portion of sea urchins) were separated using a knife, stored in ice, and immediately transported to a canning factory (Conservas y Ahumados Lou, Ribeira, La Coruña, Spain). Gonads (90 g) were placed in an RR-90 can, and then salt (750 mg) and tap water were added. The cans were vacuum-sealed and sterilized at 112°C for 50 min. Fresh gonads were also transported to the laboratory and immediately analyzed.

Reagents

Glucose standard was obtained from Merck (La Coruña, Spain) and glycogen from Sigma Chemical (Madrid, Spain). Analytical-grade citric acid from Merck and sodium carbonate from Panreac (Barcelona, Spain). HPLC-grade acetonitrile from Baker (Deventer, Holland), and demineralized water was obtained from a Milli-Q system (Millipore, Milford, MA). Freeze-dried amyloglucosidase (E.C. 3.2.1.3, from *Aspergillus niger*) was from Boehringer-Mannheim (Barcelona, Spain).

HPLC apparatus

The HPLC equipment used consisted of a Jasco (Tokyo, Japan) PU-1580 pump, a Rheodyne (Cotati, CA) 20-µL injection loop, an SP8792 column heater, and an SP4290 integrator linked via

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Labnet to a computer using Winner software version 4.00 (Spectra Physics, San Jose, CA). The RI detector was a Shodex RI-71 model. A 250- × 4.6-mm column packed with 5-µm NH_2 (Sugelabor, Madrid, Spain) was used for the separation.

Procedure

A sample (10 g) was extracted by refluxing and stirring for 4 h with 100 mL of 0.25M sodium carbonate. This mixture was cooled, adjusted to pH 4.6 with 0.25M citric acid before the addition of amyloglucosidase (20 mg, 120 units), and incubated overnight at 55–58°C to hydrolyze the glycogen to glucose. This mixture was cooled, vacuum-filtered (Whatman No. 541, Maidstone, U.K.), and increased to 100 mL with water. The resulting solution was filtered again through a 0.45-µm pore-size membrane (Millipore) and then chromatographed using an isocratic elution with an acetonitrile–water mixture (80:20, v/v) at a flow rate of 1 mL/min and the column temperature controlled at 28°C.

Results and Discussion

Development of an HPLC method for glycogen determination

To determine the efficiency of the extraction process, three preparations of the same homogenized sample of sea urchin were extracted by refluxing with 0.25M sodium carbonate for 2 (7), 4 (10), and 6 h. It was observed that the amount of glycogen extracted after 2 h was 69% of that extracted after 4 h; however, no major differences were observed in the amount of glycogen extracted between samples after 4 h and samples extracted after 6 h.

In developing the method, several changes in the proportions of the mobile phase components were attempted (85:15, 82:18, 80:20, and 78:22 acetonitrile–water) and several flow rates were tried (1, 1.2, and 1.5 mL/min). It was seen that as the amount of acetonitrile in the mobile phase was increased, the analysis time was longer. The 80:20 mixture at a flow rate of 1 mL/min allowed adequate resolution of glycogen in a shorter period of time. A system suitability test (SST version 4.1, Spectra Physics) was used to check the resolution and peak efficiency.

Figures 1 and 2 show the chromatograms of standard glucose and the glucose obtained by hydrolyzing the glycogen of the sea urchin samples, respectively. The chromatographic run time was 20 min, which included 10 min between injections to allow for the elution of interference substances.

To determine the precision of the method, six aliquots (10 g) of the same homogenized sea urchin sample were subjected to the complete procedure and injected in duplicate. The relative standard deviation (%) obtained was 3.57.

Recovery percentage was evaluated by spiking six samples of sea urchin using the method of standard addition. Samples were spiked with standard glycogen at one level (the same amount as the expected analyte amount in the sample) and then subjected to the rest of the procedure. The recovery percentage obtained was 97.2%.

The determination of the detection limit was based on the peak-to-peak noise measured on the base line close to the analyte peak (11). The value obtained for the detection limit was 40.1 µg/mL.

The method was calibrated using a series of glucose standards (four concentration levels in the range of analytical interest) measured in duplicate. Linear regression of the area of the glucose peak (y) on the concentration of the standard (x) that was obtained with 95% confidence limits yielded the equation:

$$y = 8.27x - 1.26 \qquad \text{Eq. 1}$$

where the correlation coefficient was 0.99926, the standard error of estimate was 0.50556, and the standard errors for the slope and intercept were 0.225 and 0.830, respectively.

In regards to the column life, it is well-known that an NH_2 column is very susceptible to water. However, no deterioration of the column was observed during the development of the method and the sampling carried out for this work.

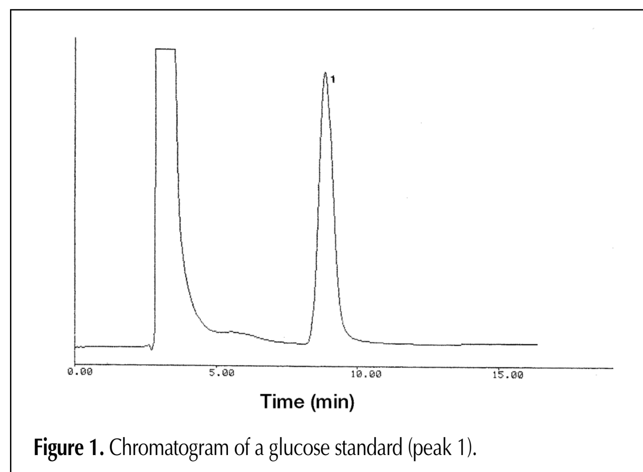

Figure 1. Chromatogram of a glucose standard (peak 1).

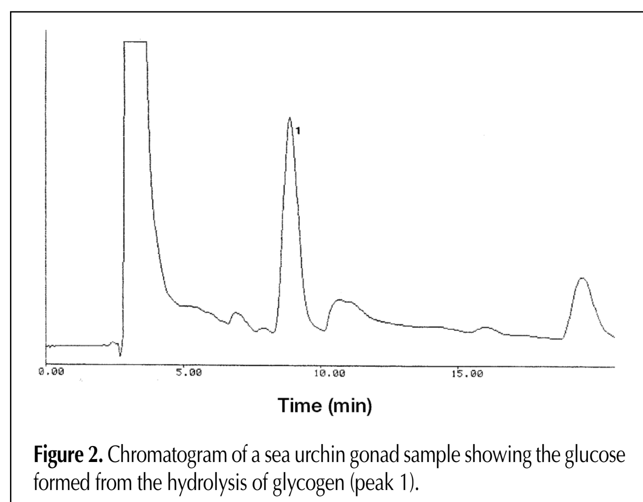

Figure 2. Chromatogram of a sea urchin gonad sample showing the glucose formed from the hydrolysis of glycogen (peak 1).

Sea urchin samples

The glycogen content of sea urchin gonads was 10.6 ± 1.47 g per 100 g dry matter (n = 10). This value was lower than that reported for sea urchins of the species *Strongylocentrotus nudus* produced in Japan, which had a glycogen content of 15.7 g per 100 g dry matter (12). This difference can be attributed to the composition of the sea urchin depending on the species (13) and its feed (14); furthermore, the glycogen content in shellfish shows seasonal variations (15).

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The glycogen content of canned gonads was 12.3 ± 2.36 g per 100 g dry matter (n = 10). Use of the t-test to compare the mean glycogen content of raw gonads and gonads sterilized at 112°C for 50 min indicated that differences were not significant ($p \leq 0.05$).

Conclusion

The results of this study suggest that HPLC separation and RI detection are useful for the determination of the glucose formed from the hydrolysis of glycogen in sea urchin gonads.

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