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Analytical Methods

Smart phone: A popular device supports amylase activity assay in fisheries research

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ABSTRACT

Colourimetric determinations of amylase activity were developed based on a standard dinitrosalicylic acid (DNS) staining method, using maltose as the analyte. Intensities and absorbances of red, green and blue (RGB) were obtained with iPhone imaging and Adobe Photoshop image analysis. Correlation of green and analyte concentrations was highly significant, and the accuracy of the developed method was excellent in analytical performance. The common iPhone has sufficient imaging ability for accurate quantification of maltose concentrations. Detection limits, sensitivity and linearity were comparable to a spectrophotometric method, but provided better inter-day precision. In quantifying amylase specific activity from a commercial source ($P > 0.02$) and fish samples ($P > 0.05$), differences compared with spectrophotometric measurements were not significant. We have demonstrated that iPhone imaging with image analysis in Adobe Photoshop has potential for field and laboratory studies of amylase.

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1. Introduction

Carbohydrate utilisation by aquatic animals is widely studied using α-amylase (EC 3.2.1.1). This enzyme is produced mainly by the pancreas and secreted into the intestine to cleave α-glycosidic links in starch. The products of this enzyme hydrolysis are maltose and maltotriose from amylose as well as glucose, maltose and dextrin from amylopectin. Activity of the amylase enzyme is useful for studies in fish feed research, such as feeding habits and growth (Thongprajukaew, Kovitvadhi, Kovitvadhi, Somsueb, & Rungruangsak-Torrissen, 2011) and digestibility of carbohydrate feedstuffs (Thongprajukaew, Kovitvadhi, Kovitvadhi, & Rungruangsak-Torrissen, 2014) as well as maturation rates in a natural ecosystem (Rungruangsak-Torrissen et al., 2012). Determinations of amylase activity with robust and low-cost equipment in the field could directly impact the feeding management in aquaculture.

Many techniques have been developed for laboratory studies of amylase activity, such as capillary isoelectric focusing (Watanabe, Yamamoto, Nagai, & Terabe, 1998), flow injection analysis with Fourier transform infrared spectroscopic detection (Schindler, Lendl, & Kellner, 1998), and lateral flow immunoassay (Bogdanovic et al., 2006). However, generally, the method used is spectroscopic determination of the hydrolysed product in 3,5-dinitrosalicylic (DNS) at 540 nm (Miller, 1959). Analysis of reducing sugars by the DNS method has been widely used for quantifying glucose levels in blood and cerebrospinal fluid (Mohun & Cook, 1962). The method is preferred because of its specificity for enzymatic reactions, such as with cellulase and α-amylase (Jurick, Vico, Whitaker, Gaskins, & Janisiewicz, 2012; Miller, 1959). However, none of these methods are suited for field studies, because of need of electric power, sensitive expensive instruments, and time-consuming protocols.

Recently, colourimetric analyses of red, green and blue (RGB) using digital camera images, processed in Adobe Photoshop, have been applied to the quantitative determination of various chemicals, such as amphetamine and methylamphetamine (Choodum & Daeid, 2011a), opiate drugs (Choodum & Daeid, 2011b), and trinitrotoluene in soil (Choodum, Kanatharana, Wongniramaikul, & Daeid, 2012).

A digital camera detects reflected light from targeted objects, and uses three different filters (red, green and blue) to enable capture of different colours using an image sensor. When the RGB layers are superimposed, an impression of colour is reproduced in the image (Choodum & Daeid, 2011a, 2011b), although the spectral characteristics are not faithfully reproduced. When digital images are analysed using programme such as Matlab (Lopez-Molinero, Linan, Sipiera, & Falcon, 2010), Kylix (Lyra et al., 2009) or Adobe Photoshop (Choodum et al., 2012), RGB intensities can be obtained,

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which represent the photons in each region of the spectrum (Cantrell, Erenas, de Orbe-Paya, & Capitan-Vallvey, 2010). The RGB data have an integer in the range 0–255 for each pixel in each channel or colour layer. The software reports RGB values 0, 0 and 0 for black and 255, 255, 255 for white image or any integer between for other colours.

Adobe Photoshop allows recovery of the separate colour layers from a digital image, and analysis of their respective intensities. Standard curves can be constructed using known concentrations of the target analyte and concentration determined based in its RGB intensities from the standard curve. Our focus was amylase activity, indicated by the concentration of maltose generated, which could be suited to this low-cost robust quantitation method. The aim of this study was to develop and elucidate the use of digital image analysis for quantifying amylase activity in fish samples. A globally popular Smart phone, iPhone, was used to collect the images. The present study could offer an easy and (relatively) rapid alternative approach to determine amylase. This method development was motivated by potential applications in aquatic farming and related field research.

2. Materials and methods

2.1. Chemicals

Maltose, soluble starch, DNS and porcine pancreatic α-amylase (13 U mg/solid) were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). Sodium chloride and sodium hydroxide were acquired from Merck Ltd. (Darmstadt, Germany) and J.T. Baker (Selangor, Malaysia), respectively. Potassium sodium tartrate was obtained from Ajax Finechem (New South Wales, Australia).

2.2. Preparation of maltose standard curve

Standard solutions were prepared by dissolving maltose in distilled water to concentrations in the range 0–80 μmol/ml. The reaction mixtures were prepared according to amylase assay described by Bernfeld (1951) with slight modifications. Briefly, 25 μl maltose was mixed with 62.5 μl phosphate buffer (0.2 mol/l, pH 8), 37.5 μl NaCl (20 mmol/l), and 125 μl Tris–HCl buffer (50 mmol/l, pH 8) containing 200 mmol/l NaCl (enzyme extraction buffer). Subsequently, the mixtures were stained by adding 250 μl DNS (1%), heated in boiling water for 5 min and then cooled to room temperature. After adding 2.5 mL distilled water, images were collected with an iPhone 4 (Model A1332, Apple Inc., California, USA).

2.3. Optical determinations of maltose

2.3.1. Measurement systems

Samples of maltose were measured using spectrophotometer (UV-200-RS, Shanghai Apogeelab Co., Ltd, China) at 540 nm and photographed with an iPhone. Images were collected with controlled illumination in a closed box (30 cm width × 30 cm length × 10 cm height) with six sample holders and a housing for the iPhone (Fig. 1). Light from external sources was blocked using opaque black corrugated plastic board whilst light intensity was maximised with a white internal background. Six light emitting diodes (lamp type) were used as the light sources.

2.3.2. Measurement methods and data collection

Six replicate samples ($n = 6$) were photographed six times. The iPhone was set to automatic focus, automatic white balance, automatic sensitivity, and single image capture mode. The images were transferred to Adobe Photoshop CS3 Extended (10.0) (Adobe

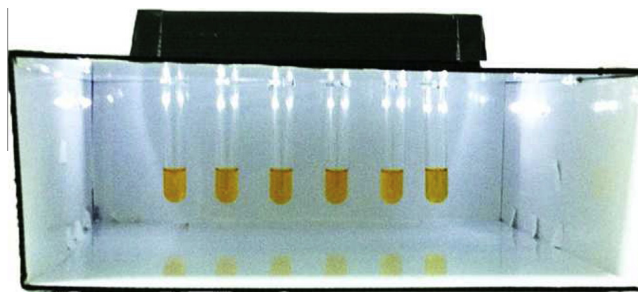

Fig. 1. Enclosure for six samples, to provide controlled illumination for iPhone photography used in quantitative determinations.

Systems, San Jose, CA). RGB colour layer intensities were obtained by selecting menu actions "crop" and "histogram". All data were subsequently transferred into Microsoft Excel 2007 (Microsoft Corp., Redmond, WA, USA) and SPSS Version 14 (SPSS Inc., Chicago, USA) for statistical analysis.

2.4. Determination of amylase specific activity in real samples

2.4.1. Preparation of enzyme extracts

Frozen small intestines from fish with different feeding habits, including carnivore (Broadhead catfish, *Clarias macrocephalus*) and omnivore (Nile tilapia, *Oreochromis niloticus*), were extracted in 50 mmol/l Tris–HCl buffer at pH 8 (1:4 w/v), using a micro-homogenizer (THP-220; Omni International, Kennesaw GA, USA). The homogenate was then centrifuged at 13,000×*g* for 20 min at 4 °C, the supernatant collected, and then kept at −80 °C for analysis of amylase. Enzyme from porcine pancreas was also dissolved in the extraction buffer before being prepared as described above.

2.4.2. Assay of amylase specific activity

The amylase specific activity in the samples was assayed according to the protocol for preparation of maltose standard curve, as described in Section 2.2, except the extraction buffer was replaced by the extracts. The extracts were assayed at pH 8, and temperatures 25 °C and 37 °C were used for samples from fish and porcine, respectively. Amylase activities from a commercial source were calculated and expressed as units (U) where 1 U gives 1 μmol maltose/min. Protein concentrations for calculating amylase specific activity (U/mg protein) from fish extracts were determined according to Lowry, Rosenbrough, Farr, and Randall (1951).

2.5. Statistical analysis

Data analyses were performed using Microsoft Office Excel 2007 and SPSS Version 14. Means and standard errors were calculated from six replicate samples ($n = 6$). Amylase activities determined using the two methods, spectrophotometer and iPhone imaging, were compared using paired sample *T*-test.

3. Results and discussion

3.1. DNS method for maltose

The DNS method has been used for colourimetric determination of maltose, a product of amylase hydrolysis. The reaction between DNS and maltose produces 3-amino-5-nitrosalicylic acid under alkaline condition (Fig. 2). This aromatic compound absorbs light strongly at 540 nm (Miller, 1959). The colours developed are orange to red-brown, depending on the concentration of maltose. However, the method is not suitable for determination of reducing sugars in complex mixtures because different sugars, generally, yield different colour intensities (Meyer, van der Wyk, & Feng, 1954).

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