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Technical note: Optimization of lactose quantification based on coupled enzymatic reactions

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ABSTRACT

A colorimetric microplate-adapted lactose assay was developed to quantify lactose in dairy products. The assay was based on the coupled enzymatic reaction of β-galactosidase-glucose oxidase-horseradish peroxidase using Amplex red as detection probe. The assay showed good linearity in the range of 0.1 to 0.5 mmol of lactose/L, with a limit of detection of 0.0433 mmol/L and a limit of quantification of 0.1313 mmol/L. The lactose assay at optimized conditions (5 U of β-galactosidase/mL, 5 U of glucose oxidase/mL, 1 U of horseradish peroxidase/mL, and 100 μmol of Amplex red/L for 1 h at 37°C in the dark) showed good correlation with a commercial lactose enzymatic kit with intraassay variation below 10% and interassay variations below 7.6%. The developed lactose microplate assay can be adopted as routine analysis for lactose determination in dairy products due to its relatively low cost compared with a commercial kit, relatively short reaction time, and high sensitivity and reproducibility.

Key words: lactose, β-galactosidase, enzymatic reaction

Technical Note

Lactose is a disaccharide sugar derived from galactose and glucose, which form a β-1→4 glycosidic linkage. Intestinal absorption assimilation of this disaccharide requires hydrolysis to free glucose and galactose, a reaction catalyzed by lactase. Lactose intolerance in consumers with low lactase activity has promoted the development of a market for low-lactose or lactose-free products. Therefore, a protocol that can be implemented for routine analysis will have extensive application for analysis of lactose in dairy products.

Several analytical methods to quantify lactose have been published, including spectrophotometric (Harris, 1986; Shapiro et al., 2002; Fornera et al., 2011), chromatographic (Xinmin et al., 2008; Erich et al., 2012), electrochemical methods using 5-aminosalycilic acid (Eshkenazi et al., 2000) or tetrathiafulvalene as probes to detect amperometric signals (Conzuelo et al., 2010), and a method that quantifies lactose indirectly by measuring the glucose released after enzymatic hydrolysis using a blood glucose biosensor (Amamcharla and Metzger, 2011). However, the chromatographic and electrochemical methods require instruments that are not usually available in laboratories for routine analysis.

The enzymatic cascade spectrophotometric method quantifies the glucose released from lactose by β-galactosidase and oxidized by glucose oxidase, which produces hydrogen peroxide (H_2O_2) that oxidizes the *o*-phenylenediamine detection probe (Fornera et al., 2011). However, the reaction requires 7 h, which makes the procedure time consuming and not practical for routine analysis.

Amplex red (**AR**) is a colorless and nonfluorescent compound that is commonly used as a probe for the measurement of extracellular H_2O_2 in biological systems. Amplex red reacts with H_2O_2 in 1:1 stoichiometry to form colored and fluorescent resorufin, catalyzed by horseradish peroxidase (Rhee et al., 2010). Amplex red-based commercial assay kits have been developed for multiple analysis including glucose, galactose (Invitrogen Corp., Carlsbad, CA), and lactose (BioVision Inc., Milpitas, CA), which quantifies lactose through the galactose released. Our aim was to optimize a lactose assay based on the coupled enzymatic reactions using β-galactosidase-glucose oxidase-horseradish peroxidase and AR as detection probe to be implemented as routine analysis.

β-Galactosidase (**β-Gal**) from *Escherichia coli* grade VIII (G5635-1KU), glucose oxidase (**GOD**) from *Aspergillus niger* type VII (G2133-10KU), AR (90101-5MG-F), dimethyl sulfoxide (D8418-100ML), D-lactose monohydrate (61339-25G), horseradish peroxidase (**HRP**) type XII (P8415-500UN), magnesium chloride anhydrous (M8266-100G), sodium chloride (S7653-250G), and sodium phosphate monobasic dehydrate (71505-250G) and dibasic (S7907-100G) were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). The lactose enzymatic kit (K624-100) was purchased from BioVision Inc.

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Reduced-fat milk, lactose-free milk, powder nonfat milk, and plain Greek yogurt were purchased from a local supermarket in Pullman, Washington. Whey from the American Cheddar cheese (Cougar Gold) and the Monterey Jack-style non-Cheddar cheese (Viking) processing were provided by the creamery at Washington State University (Pullman). Whey was subjected to UF (Romicon model HF-LAB-5; Koch Membrane Systems Inc., Wilmington, MA) to obtain whey permeate containing lactose and other sugars with a polyethersulfone membrane (10,000-Da cutoff). Powder milk and Greek yogurt were homogenized with water (1:10); a 1-mL aliquot of sample was then mixed with 100 μL of 20% (wt/vol) cold TCA and centrifuged at 21,130 × *g* for 5 min at 4°C to eliminate proteins. Supernatants were stored at −20°C until analysis.

Serial dilutions (0.1 to 0.5 mmol/L) of D-lactose were prepared from stock solution (10 mmol/L). A stock solution of β-Gal (1,000 U/mL) was prepared in PBS (50 mmol/L, pH 7.2) containing $MgCl_2$ (2 mmol/L), GOD (1,000 U/mL), and HRP (500 U/mL). Amplex red was prepared in dimethyl sulfoxide at 10 mmol/L. All solutions were stored at −20°C.

The spectrum of absorption of resorufin produced by oxidation of AR at reaction conditions (20 U of β-Gal/mL, 5 U of GOD/mL, 1 U of HRP/mL, and 100 μmol of AR/L at 37°C for 30 min in the dark, with 0.1 to 0.5 mmol of lactose standard/L) was monitored between 400 and 700 nm every 5 nm using an μQuant microplate reader (BioTek Instruments Inc., Rochester, VT) to determine the wavelength for maximum absorption.

The hydrolysis of lactose by β-Gal is the first enzymatic reaction and limiting step in the lactose assay. Lactose (0.5 mmol/L) was hydrolyzed by β-Gal at 5, 10, and 20 U/mL in the presence of GOD (5 U/mL), HRP (1 U/mL), and AR (100 μmol/L) at 37°C for 120 min. The absorbances were monitored at 570 nm from 30 to 120 min.

Ten microliters of lactose standard (0.1 to 0.5 mmol/L) was mixed with 100 μL of AR reagent (**ARR**) containing β-Gal (5 U/mL), GOD (5 U/mL), HRP (1 U/mL), and AR (100 μmol/L) dissolved in PBS on a 96-well plate. Absorbances at 570 nm were monitored every 2 min in reactions incubated at 20 and 37°C for 30 min. Linear regression analysis was used to calculate the slopes of each standard curve and slope values were used to compare effects of time and temperature on the reaction.

Dietary antioxidants at biological concentrations produce interferences in the AR/HRP catalyzed reaction (Serrano et al., 2009) that might result in overquantification of lactose. Dairy products were assessed for interferences due to the presence of H_2O_2 or antioxidant activity that can catalyze AR-HRP reactions. Briefly, samples were diluted in PBS (1:600 for milk and lactose-free milk, 1:1,000 for powder milk, 1:100 for Greek yogurt, and 1:300 for whey cheese permeates); a 10-μL sample or PBS (blank) was mixed with 100 μL of AR (100 μmol/L) and HRP (1 U/mL). Absorbances at 570 nm after incubation at 37°C for 1 h were compared with the blank.

In addition, antioxidant activities of samples were assessed by the $ABTS^{\bullet+}$ assay as previously reported (Re et al., 1999). Briefly, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (**$ABTS^{\bullet+}$**) produced by reaction of 7 mmol of ABTS/L with 2.45 mmol of potassium persulfate/L for 16 h at room temperature in the dark was diluted with PBS to reach an absorbance of approximately 0.80 at 405 nm. A 10-μL aliquot of diluted sample or Trolox (0–300 μmol/L) and 200 μL of $ABTS^{\bullet+}$ working solution were added to a 96-well plate and incubated for 6 min at room temperature. Antioxidant activity was monitored at 405 nm against the standard curve of Trolox and expressed as μmol of Trolox equivalent/L.

To evaluate the effect of initial glucose concentration in diluted samples, a stock solution of ARR without β-Gal (**$ARR^{-/-}$β-Gal**) was used. Lactose content in the samples was calculated by subtracting the absorbance obtained in the $ARR^{-/-}$β-Gal reaction from the absorbance obtained with the ARR reaction.

Standard curves of lactose were constructed to evaluate the linearity of the enzymatic assay. Reactions were carried out with lactose standard (0.1 to 0.5 mmol/L) and 100 μL of ARR stock solution at 37°C for 1 h. The linearity was evaluated by the coefficient of determination and residual plot obtained from regression analysis. The sensitivity of the assay was evaluated by the limit of detection (**LOD**) and limit of quantification (**LOQ**). The LOD and LOQ were calculated from standard curves (Shewiyo et al., 2012) as follows: LOD $= 3.3\sigma/S$ and LOQ $= 10\sigma/S$ where σ was the standard deviation for absorbance of the blank ($n = 3$) and S was the slope of the standard curve.

The intraassay and interassay variations were evaluated through the relative standard deviation (**RSD**) percentage. For intraassay variations, 3 replicates of 5 different samples were run in 1 d and RSD percentage = (SD/mean of each sample) × 100. For interassay variations, the same sample was analyzed on 3 different days and RSD percentage = (SD/mean value of 3 d) × 100. A lactose enzymatic commercial assay kit (Lab Vision Corp., Fremont, CA) was used to validate our lactose assay.

Ten microliters of sample or lactose standard (0.1–0.5 mmol/L) and 100 μL of ARR or $ARR^{-/-}$β-Gal were mixed for 15 s on a 96-well plate using a titer plate shaker at speed = 6 (Thermo Scientific, Asheville, NC)

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