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A Simple Enzymatic Method for Quantitation of 2′-Fucosyllactose

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2′-Fucosyllactose (2′-FL) is one of the most important human milk oligosaccharides and has several health benefits for infants. The levels of 2′-FL in breast milk or samples from other sources can be quantified by high-performance liquid chromatography. However, this method cannot be used for simultaneous detection of the target compound in numerous samples. Here, we developed a simple method for quantifying 2′-FL in a microplate format. The method involves two steps: (i) release of L-fucose from 2′-FL by α-(1-2,3,4,6)-L-fucosidase and (ii) measurement of NADPH formed during the oxidation of L-fucose by L-fucose dehydrogenase. This method enables measurement of up to 5 g/l 2′-FL in 50 min using a 96-well microplate. The efficiency and simplicity of the proposed method make it suitable for the analyses of a large number of samples simultaneously.

Keywords: 2′-Fucosyllactose, quantitation, α-(1-2,3,4,6)-L-fucosidase, L-fucose, L-fucose dehydrogenase

Introduction

2′-Fucosyllactose (2′-FL) has been found to be the most abundantly represented human milk oligosaccharide (HMO) in breast milk of secretor mothers [1]. In the molecule of 2′-FL, the fucosyl residue is attached to lactose through an α-1,2-glycosidic linkage [2]. In addition to their role as prebiotics, fucosylated HMOs with terminal α-1,2-fucosyl linkages are reported to exhibit protective activity against several pathogens, including *Campylobacter jejuni*, enterotoxigenic *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Candida albicans*, and noroviruses [3–7]. Clinically, quantitative variations in 2′-FL may affect the capacity of human milk to protect a nursing infant [8]. Low levels of this fucosyloligosaccharide in the milk of sore mothers have been reported to be associated with a higher rate of diarrhea in breast-fed infants [3]. Furthermore, HMOs in breast milk from more than 20% of women are characterized by the absence of 2′-FL [9]. The potential nutraceutical and pharmaceutical applications of 2′-FL necessitate its mass production.

Fast screening of highly active 2′-FL-producing variants from a large mutant library using a simple quantitation method is crucial for the development of more efficient production of this important HMO in a suitable amount at a reasonable price. Furthermore, the measurement of 2′-FL contents in a variety of samples is a basic analytical operation in many phases of its biotechnological production process. At present, the concentration of 2′-FL in samples of different origin can be quantified using several methods, such as high-pH anion-exchange chromatography, HPLC, and LC-MS [10–14]. However, these methods are time-consuming and can be challenging for a large number of samples.

α-L-Fucosidases (E.C. 3.2.1.51) are exoglycosidases capable of cleaving α-linked L-fucose residues from fucosyloligosaccharides [15]. As a result of defucosylation, fucose is released from these glycoconjugates. This useful property of α-L-fucosidases allows for their possible use as efficient biochemical tools for the quantification of HMOs in various samples. In agreement with this idea, the purpose of the present study was to develop a convenient enzymatic method for the quantitation of 2′-FL in analyzed samples. The two-step approach proposed in this study aims to combine (i) 2′-FL cleavage by α-(1-2,3,4,6)-L-fucosidase from *Homo sapiens* (FUCHS) and (ii) measurement

of the released L-fucose amounts. Based on the amount of L-fucose released, the concentration of 2′-FL in the sample can be determined.

Materials and Methods

Reagents and Apparatus

The FUCHS and L-fucose assay kit were purchased from Megazyme (Ireland). 2′-Fucosyllactose was obtained from AP Technology (Korea). Other chemicals were of analytical grade. A microplate reader (SpectraMax M2) and its accompanying software (Soft Max Pro 5) were from Molecular Devices (USA). The HPLC system was from Waters Corporation (USA).

Standard and Sample Preparation

Standard stock solution (20 mM) containing 2′-FL was diluted with ultrapure water to concentrations of 1–20 mM. The stock solution of the standard mixture containing 2′-FL (10 mM), lactose (55.5 mM), and glycerol (271.5 mM) was also diluted with ultrapure water to yield different concentrations of these compounds. Each tube was mixed thoroughly.

For sample preparation, an aliquot (0.5 ml) of the fermentation sample containing extracellular 2′-FL was incubated at 95°C for 5 min to inactivate the enzyme activity and then centrifuged at 10,000 ×*g* for 10 min at 4°C. The clear supernatant was used for the 2′-FL cleavage reaction.

Determination of Optimal Parameters for 2′-FL Cleavage

After preparation of all reagents and working standards, the temperature for complete 2′-FL cleavage, substrate (2′-FL) concentration, and amount of FUCHS were optimized as follows. 2′-FL cleavage was conducted using FUCHS. This enzyme is known to be most active at pH 4 (FUCHS datasheet). To investigate the temperature dependence of FUCHS for complete 2′-FL cleavage, enzymatic reactions were conducted at 40°C, 45°C, and 50°C for 30 min. The enzyme was diluted in 100 mM sodium acetate buffer (pH 4.0) containing 1 mg/ml bovine serum albumin. The effects of FUCHS on 2′-FL were determined by incubating 100 mU of this enzyme with 2 µl of 2′-FL at a substrate concentration of 0.2 mM in a 20-µl reaction volume. Reactions were stopped by increasing the pH value to 9.5. To determine the optimum substrate concentration, 2′-FL (0.1–2 mM) was coincubated with a constant amount of enzyme (100 mU) in a total reaction volume of 20 µl for 30 min at an appropriate temperature. The optimal amount of FUCHS for 2′-FL cleavage was determined by incubating different amounts of it (50–200 mU) with 2′-FL for 30 min. Control reactions without substrate or enzyme were also carried out. To quantify the 2′-FL concentrations in the samples, L-fucose released during substrate hydrolysis was measured.

Determination of 2′-FL Concentration by HPLC

Before cleavage, the amounts of extracellular 2′-FL in the samples were determined by an HPLC system equipped with a Rezex ROA Organic Acid H^+ column (Phenomenex, USA) and refractive index detector. The column was eluted with 0.01 N H_2SO_4 at a flow rate of 0.6 ml/min at 50°C [10]. The completeness of 2′-FL cleavage by FUCHS was confirmed by measuring its residual concentration after the enzymatic reaction using this method.

Determination of L-Fucose Concentration

The amount of L-fucose generated by the 2′-FL cleavage reaction was determined using an L-fucose assay kit, following a modified microplate assay procedure. First, the reaction components without L-fucose dehydrogenase (FDH) were mixed thoroughly. After 4 min, the absorbance (A1) at 340 nm was determined using the microplate reader SpectraMax M2. The reaction was started by the addition of FDH (5 µl). At the end of the reaction (after 20 min), the absorbance (A2) at 340 nm was measured again. An L-fucose calibration curve was constructed using L-fucose standard solutions (0.06–3 mM). The blank OD_{340} was subtracted from the standard OD_{340} values. The absorbance difference was plotted against the standard concentrations. Next, the slope was determined by linear regression fit of the standard points. The concentration of L-fucose in analyzed samples was calculated on the basis of the calibration curve. Statistical data analysis was conducted using GraphPad Prism 5 (GraphPad Software, Inc., USA).

Results and Discussion

Proposed Scheme for Enzymatic Detection of 2′-FL

2′-FL performs several biological functions important for infant health [2, 4, 9, 16]. Various methods for the quantitation of 2′-FL in samples have been described [10–14]. However, these methods are time-consuming and labor-intensive. L-Fucose is a common monosaccharide present at the nonreducing end of fucosylated HMOs [2, 15]. Based on previous reports, α-L-fucosidases are useful for studying many fucose-containing oligosaccharides and polysaccharides [17, 18]. In particular, α-L-fucosidase from *Pecten maximus* is considered useful for studying the structural properties of fucoidan [17]. Furthermore, α-L-fucosidase can be utilized to evaluate the biological activities of fucose-containing glycoconjugates [18]. In addition to these applications, several known α-L-fucosidases of different origin can hydrolyze fucosylated HMOs, including 2′-FL [19–21]. Released L-fucose can be oxidized by the enzyme FDH (E.C. 1.1.1.122) in the presence of nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to L-fucono-1,5-lactone with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (Fig. 1). The amount of NADPH formed in the second reaction is stoichiometric with the amount of L-fucose and can be measured as the increase in absorbance at 340 nm. In the

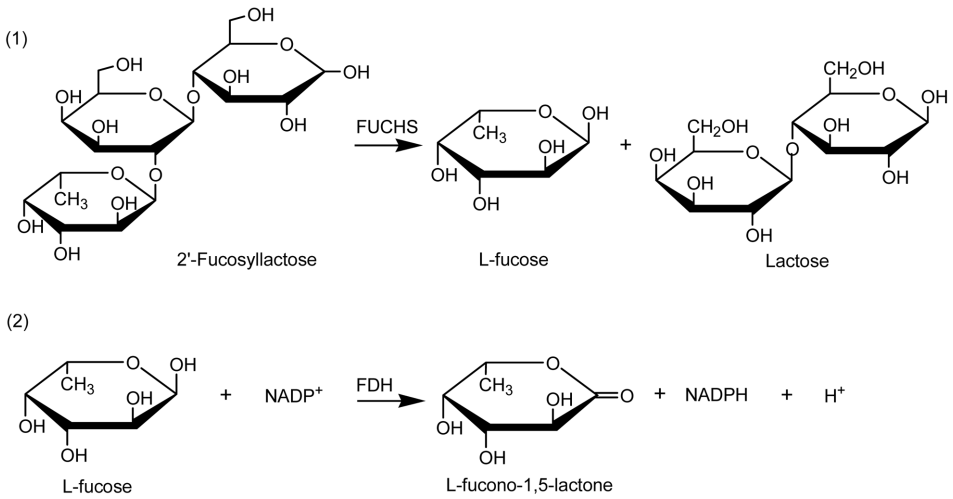

Fig. 1. Proposed scheme of the method for 2′-fucosyllactose quantitation.
FUCHS, α-(1-2,3,4,6)-L-fucosidase from Homo sapiens; FDH, L-fucose dehydrogenase.

present study, the amount of L-fucose, generated after the 2′-FL cleavage reaction, was measured using an L-fucose assay kit. The reaction components are listed in Table 1. The amount of 2′-FL in each sample was quantified on the basis of the concentration of enzymatically released L-fucose, which was determined using a calibration curve (Fig. S1).

Optimization of Parameters for 2′-FL Cleavage

FUCHS is known to be most active at pH 4 (FUCHS datasheet). The temperature dependence of FUCHS was determined with respect to its hydrolytic activity to 2′-FL. The optimum temperature reported for this enzyme is 50°C (FUCHS datasheet). However, because of measurement problems caused by the limited temperature control options of some microplate readers, lower temperatures (40°C and 45°C) were also tested. As a result, an optimal activity of FUCHS for efficient 2′-FL cleavage was observed at 50°C (Fig. 2). The enzyme showed considerably lower activity at 40°C. Therefore, to achieve complete cleavage of 2′-FL at 40°C, either the time of the enzymatic reaction must be lengthened or the enzyme concentration should be increased. This illustrates that FUCHS activity is limited by its narrow temperature range. A temperature of 45°C was sufficient to promote complete cleavage of 0.2 mM 2′-FL by the enzyme in the reaction mixture (Fig. 2). These results were confirmed by HPLC (data not shown).

Fig. 3A shows the effect of substrate (2′-FL) concentrations on 2′-FL cleavage by 100 mU FUCHS at 45°C and 50°C.

Table 1. Components of the L-fucose assay reaction mixture.

Reaction component	Blank (µl)	Standard (µl)	Sample (µl)
Distilled water	105	100	100
Standard solution	-	5	-
Sample solution	-	-	5
Solution 1 (buffer)	20	20	20
Solution 2 ($NADP^+$)	5	5	5
Suspension 3 (FDH)	5	5	5
Total reaction volume (µl)	135	135	135

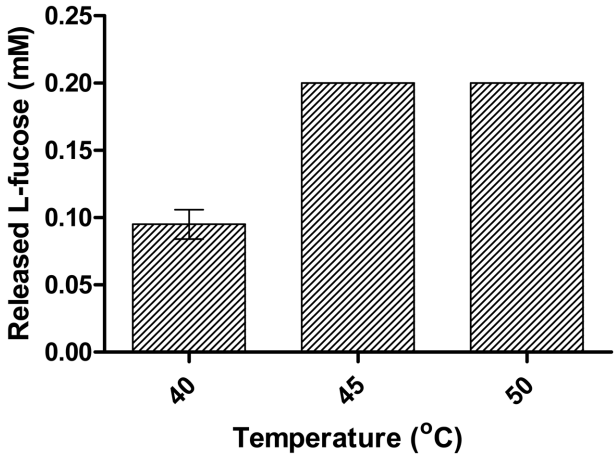

Fig. 2. Temperature dependence of L-fucosidase from *Homo sapiens* (FUCHS) activity for 2′-fucosyllactose (2′-FL) cleavage.
The amount of 2′-FL was determined based on L-fucose released after cleavage of 0.2 mM 2′-FL by 100 mU FUCHS at different temperatures for 30 min.

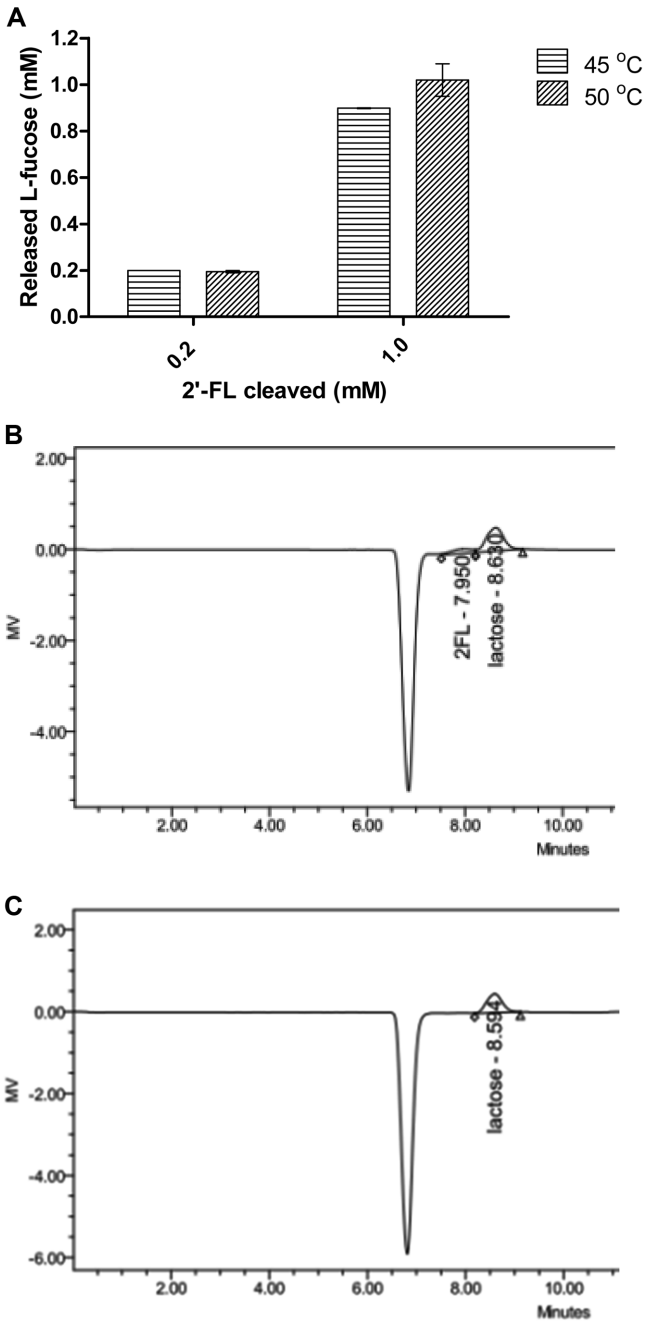

Fig. 3. L-Fucose released during cleavage with varying 2′-fucosyllactose (2′-FL) concentrations.
2′-FL was cleaved by 100 mU FUCHS at 45°C and 50°C (**A**). Reaction products after cleavage of 1 mM 2′-FL at 45°C (**B**) and 50°C (**C**) for 30 min were analyzed by HPLC.

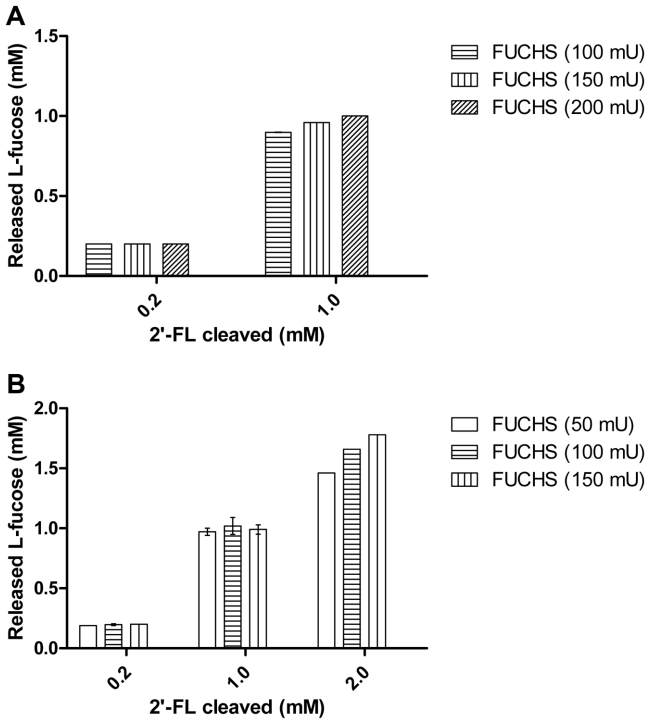

Fig. 4. Release of L-fucose after cleavage of 2′-fucosyllactose (2′-FL) by different amounts of FUCHS.
2′-FL was cleaved by varying amounts of FUCHS at 45°C (**A**) and 50°C (**B**) for 30 min. FUCHS, L-fucosidase from *Homo* sapiens.

Cleavage of 1 mM 2′-FL in the reaction mixture was completed only at 50°C. This was also confirmed by HPLC (Fig. 3C). However, complete cleavage of 2 mM 2′-FL in the reaction mixture by 100 mU of FUCHS was not achieved at 50°C (Fig. S2).

Fig. 4 demonstrates the effect of different amounts of FUCHS on 2′-FL cleavage. For complete cleavage of 0.2 mM 2′-FL in the reaction mixture, 100 and 50 mU of enzyme were sufficient at 45°C and 50°C, respectively (Fig. 4). To complete cleavage of 1 mM 2′-FL in the reaction mixture at 45°C, 200 mU of FUCHS was required (Fig. 4A), whereas at 50°C, 100 mU of this enzyme was sufficient (Fig. 4B). This was confirmed by HPLC (data not shown). However, efficient cleavage of 2 mM 2′-FL was not achieved even with 150 mU of FUCHS at 50°C (Fig. 4B).

Effects of Lactose and Glycerol on 2'-FL Cleavage

Breast milk contains lactose [22]. Furthermore, when 2′-FL is produced by metabolically engineered microbial strains, glycerol and lactose are used as a carbon source and receptoracceptor for the fucosyl residue, respectively [23]. Therefore, the interference of lactose and glycerol in the reaction mixture on 2′-FL cleavage by FUCHS was also investigated. Table 2 shows the concentrations of 2′-FL,

Table 2. Tested concentrations of 2′-fucosyllactose (2′-FL), lactose, and glycerol in the reaction mixtures.

Level of standard mixture	2′-FL (mM)	Lactose (mM)	Glycerol (mM)
1	0.25	1.39	6.79
2	0.50	2.77	13.57
3	1.00	5.55	27.15

lactose, and glycerol used for analyses. Lactose and glycerol did not interfere with 2′-FL cleavage by FUCHS both at 45°C and 50°C (Fig. 5).

2′-FL Cleavage in Fermentation Samples

Fig. S3 demonstrates the results of 2′-FL cleavage in fermentation samples by FUCHS at 45°C and 50°C. Initial amounts of extracellular 2′-FL in the samples were determined by HPLC as previously described [10].

Fig. 6 shows good correlation between the initial concentrations of 2′-FL in samples quantified by HPLC and amounts of 2′-FL determined based on released L-fucose measurements after enzymatic cleavage of 2′-FL.

Based on our results, the following recommendations were made. In microplate readers with a temperature control of up to 65°C, to achieve complete cleavage of up to 10 mM 2′-FL at 50°C without sample dilution, 100 mU of FUCHS can be used. If the sample is initially diluted by up to 2 mM 2′-FL, 50 mU FUCHS is sufficient at the same temperature. However, in microplate readers with temperature control of up to 45°C, for complete cleavage of up to 10 mM 2′-FL at 45°C without sample dilution, 200 mU FUCHS is recommended. If the sample is initially diluted up to 2 mM 2′-FL, 100 mU FUCHS is sufficient.

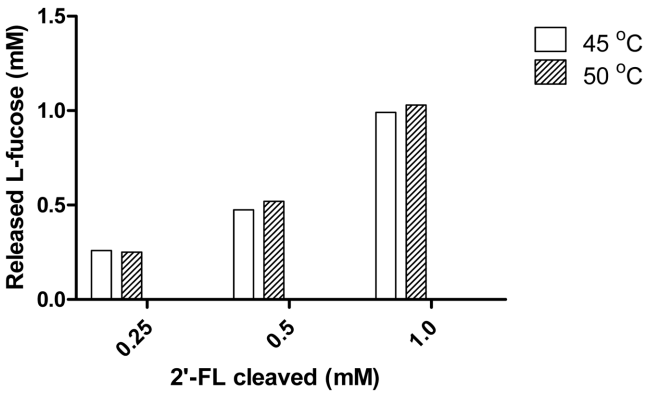

Fig. 5. 2′-Fucosyllactose (2′-FL) cleavage by FUCHS in the presence of lactose and glycerol.
2′-FL was cleaved by 200 mU FUCHS at 45°C and 100 mU FUCHS at 50°C for 30 min. FUCHS, L-fucosidase from *Homo sapiens*.

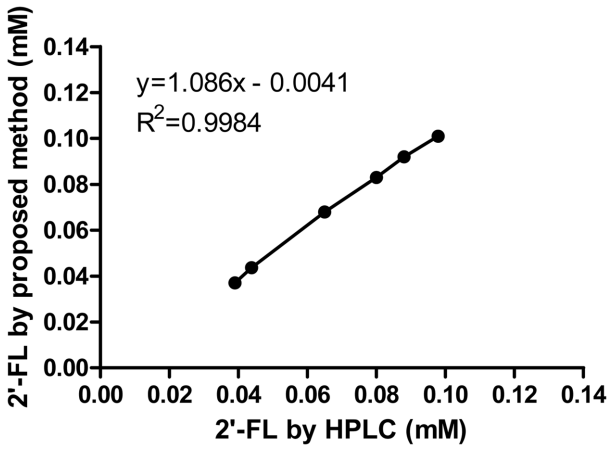

Fig. 6. Correlation of 2′-fucosyllactose amounts determined in fermentation samples by HPLC and the present method.

The method proposed in this study has some limitations. If the sample generates values higher than the highest standard of L-fucose used to construct the calibration curve, dilution of the sample is required. Any variation in pipetting technique, incubation time, or temperature can cause variation in the results. In addition to 2′-FL, FUCHS can cleave a few other HMOs present in samples, although this reaction is extremely slow.

In summary, the amounts of 2′-FL in various samples are currently determined by lengthy analyses. These methods cannot quantify this HMO in a large number of samples simultaneously. As expected, FUCHS cleaved L-fucose from 2′-FL very efficiently. These results demonstrate the applicability of our developed enzymatic method for quantitation of 2′-FL in a microplate format in a time-efficient manner. This method can be used for rapid screening of active variants during the development of microbial strains producing 2′-FL. Furthermore, the method is useful for measuring 2′-FL contents in a variety of samples analyzed during different phases of the biotechnological production process. The approach used in this study might also be applicable for enzymatic detection of 2′-FL in breast milk, although additional experiments using human milk are needed to confirm these results.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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