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A simple enzymatic method for quantitation of 2′-fucosyllactose

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Running title: A simple method for quantitation of 2′-fucosyllactose
Abstract

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: (1) release of L-fucose from 2′-FL by α-(1-2,3,4,6)-L-fucosidase and (2) measurement of NADPH formed during 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 makes 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, fucosyl residue is attached to lactose through an α-1,2-glycosidic linkage [2]. In addition to the 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 variation in 2′-FL may affect the capacity of human milk to protect a nursing infant [8]. Low levels of this fucosyl-oligosaccharide 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]. Nutraceutical and pharmaceutical potential applications of 2′-FL necessitate its mass production.

Fast screening of highly-active 2′-FL-producing variants from a large mutant library using 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, 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 (HPAEC), 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 (EC 3.2.1.51) are exoglycosidases capable of cleaving α-linked L-fucose residues from fucosyl-oligosaccharides [15]. As a result of defucosylation, fucose is released from these glycoconjugates. This useful property of α-L-fucosidases can make it possible to use these enzymes as efficient biochemical tools for quantification of HMOs in various samples. In agreement with this idea, the purpose of the present study is to develop a convenient enzymatic method for quantitation of 2'-FL in analyzed samples. Two-step approach, proposed in this study, aims to combine (1) 2'-FL cleavage by α-(1-2,3,4,6)-L-fucosidase from *Homo sapiens* (FUCHS) and (2) 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 (Wicklow, Ireland). 2'-Fucosyllactose was obtained from AP Technology (Suwon, Korea). Other chemicals were of analytical grade. A microplate reader (SpectraMax M2) and its accompanying software (Soft Max Pro 5) were from Molecular Devices (Sunnyvale, CA, USA). The HPLC system was from Waters Corporation (Milford, MA, 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 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 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 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 samples were determined by HPLC equipped with a Rezex ROA Organic Acid H⁺ column (Phenomenex, Torrance, CA, USA) and refractive index (RI) detector. The column was eluted with 0.01 N H₂SO₄ 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 OD₃₄₀ was subtracted from the standard OD₃₄₀ values. The absorbance difference was plotted against standard concentrations. Next, the slope was determined by the linear regression fit of standard points. The concentration of L-fucose in analyzed samples was calculated based on the calibration curve. Statistical data analysis was conducted using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, 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 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 (EC 1.1.1.122) in the presence of nicotinamide-adenine dinucleotide phosphate (NADP⁺) to L-fuco-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 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 based on 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).

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

Figure 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 was 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), while 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).
Effect of lactose and glycerol

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 receptor 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, 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

Figure 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 samples were determined by HPLC as previously described [10].

Figure 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.

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 be also applicable for enzymatic detection of 2′-FL in breast milk, although additional experiments using human milk are needed to confirm these results.

Acknowledgements

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Conflict of interest

All authors declare that they have no conflict of interest.

Appendix A. Supplementary material

References


are associated with protection against diarrhea in breast-fed infants. *J. Pediatr.* **145:** 297-303.


18. Sakurama H, Tsutsumi E, Ashida H, Katayama T, Yamamoto K, Kumagai H. 2012. Differences in the substrate specificities and active-site structures of two alpha-L-


Figure legends

**Fig. 1. Proposed scheme of the method.** The names of the enzymes are abbreviated as follows: FUCHS, α-(1-2,3,4,6)-L-fucosidase from *Homo sapiens*; FDH, L-fucose dehydrogenase.

**Fig. 2. Temperature dependence of FUCHS activity for 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′-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. The release of L-fucose after cleavage of 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.

**Fig. 5. 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.

**Fig. 6. Correlation between 2′-FL amounts determined in fermentation samples by HPLC and the present method.**
**Tables**

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

<table>
<thead>
<tr>
<th>Reaction component</th>
<th>Blank (µL)</th>
<th>Standard (µL)</th>
<th>Sample (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>105</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Standard solution</td>
<td>-</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Sample solution</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Solution 1 (buffer)</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Solution 2 (NADP⁺)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Suspension 3 (FDH)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>135</td>
<td>135</td>
<td>135</td>
</tr>
</tbody>
</table>
Table 2. Tested concentrations of 2′-FL, lactose, and glycerol in the reaction mixtures

<table>
<thead>
<tr>
<th>Level of standards</th>
<th>2′-FL (mM)</th>
<th>Lactose (mM)</th>
<th>Glycerol (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mixture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.25</td>
<td>1.39</td>
<td>6.79</td>
</tr>
<tr>
<td>2</td>
<td>0.50</td>
<td>2.77</td>
<td>13.57</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>5.55</td>
<td>27.15</td>
</tr>
</tbody>
</table>
Figures

Figure 1

(1) 2'-Fucosyllactose → L-fucose + Lactose

(2) L-fucose + NADP⁺ → L-fucono-1,5-lactone + NADPH + H⁺
Figure 2

![Bar chart showing released L-fucose (mM) at different temperatures (°C).](image-url)
Figure 3

A

B
lactose - 8.59g
Figure 4

A

B

[Graph A: Release of L-fucose vs. 2'-FL cleaved for different FUCHS concentrations]

[Graph B: Release of L-fucose vs. 2'-FL cleaved for different FUCHS concentrations]
Figure 5
Figure 6

\[ y = 1.086x - 0.0041 \]

\[ R^2 = 0.9984 \]