Cellular and Molecular Biology

Research Article Open Access

Construction of a Glucose Biosensor Using the *fbp1 – GFP* Reporter System in the Fission Yeast *Schizosaccharomyces pombe*

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Abstract

Fission yeast (*Schizosaccharomyces pombe*) is used as a platform for analyzing the activity of heterologous G protein-coupled receptors (GPCR). This type of yeast has two GPCR systems; one for detecting pheromones, and another for detecting glucose. We previously reported on a green fluorescent protein (GFP) reporter system in fission yeast that evaluated ligand concentrations by measuring the activity of endogenous pheromones. In this paper, we describe the use of the GFP reporter system as a glucose receptor assay. We engineered yeast to express reporter plasmids in which the fbp1 promoter was fused with GFP. The expression of Fbp1 is inhibited by glucose, so the transformed cells expressed high levels of GFP in the absence of glucose. When the transformed yeast cells were cultured in varying concentrations of glucose, the level of GFP expression was dependent on ligand (glucose) concentration. This method enabled 10 μM glucose to be measured, and could be used as a glucose biosensor.

Keywords: GPCR Biosensor; GFP; Fission yeast; Glucose

Introduction

G protein-coupled receptors (GPCRs) are integral cell-membrane proteins that share a common structure of seven transmembrane helices [1]. GPCRs bind extracellular ligands and transmit the signal to intracellular signaling pathways, and as such play an important role in cell communication. GPCRs are coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins), which consist of three subunits: α, β, and γ. When a GPCR binds a ligand, the Gα subunit dissociates from the Gβγ subunit and transmits signals to downstream pathways [2-5]. GPCRs respond to a variety of stimuli such as neurotransmitters, hormones, light, odors, and taste [4,5]. To recognize such various external stimuli, there are about 800 members of the GPCR superfamily in humans, which are grouped into five major classes [6,7]. These receptors are the primary targets for many prescription drugs; over 30% of approved and marketed drugs target GPCRs [8-10]. However, there are about 100 orphan GPCRs for which ligands have not yet been identified [9,11]. Identifying the ligands that bind to these orphan GPCRs will not only improve our knowledge of orphan GPCRs, their signaling pathways, and physiological function but also aid research on disease and facilitate drug discovery efforts.

Fission yeast (*Schizosaccharomyces pombe*) is a free-living eukaryote that shares many features with cells from more complicated eukaryotes [12]. Fission yeast can be easily genetically manipulated and is one of the most popular experimental organisms for studying cell-cycle control, mitosis and meiosis, DNA repair and recombination, and the checkpoint controls important for genome stability [13-15]. The fission yeast has two GPCR-mediated signaling pathways, a pheromone signaling pathway and a glucose signaling pathway [16]. The glucose signaling pathway in fission yeast senses extracellular glucose using the Git3 glucose receptor [17]. When nutrient glucose is adequately supplied, the glucose signal is transmitted from Git3 to the Gα protein Gpa2. Activated Gpa2 then stimulates the adenylate cyclase Cyr1 [16-19]. Increased cAMP activates protein kinase A (PKA), which suppresses the transcription of genes involved in gluconeogenesis and sexual development, including fbp1, which encodes the gluconeogenic enzyme fructose-1, 6-bisphosphatase [17,19,20]. Changes in the expression of fbp1 therefore present an opportunity for monitoring glucose levels.

Glucose is an important biological molecule that is used as a cellular energy source. Blood glucose levels vary before and after meals even in healthy people. The pre-meal glucose value is usually about 5 mM [21]. Detecting glucose levels is important for medical and diagnostic purposes, especially for individuals with diabetes. Abnormally high blood sugar levels can cause apoptosis and inflammation [22,23]. To develop new ways to monitor glucose levels, we constructed an fbp1–GFP reporter gene assay that monitors the response to glucose levels based on GFP readout. The method developed here can detect 10 μM glucose; this detection sensitivity is comparable to existing high sensitivity ones [24].

Materials and Methods

Yeast strains and growth media

The strains used in this study are listed in Table 1. The OSP260 strain was created by knocking-out gpa1, gpa2, and git3 from the OSP230 strain [25]. The strain created by introducing Pfbp1–GFP downstream of the glucose signaling pathway of OSP260 was designated as the FGFP strain. An FGFP (Gpa2+) strain was prepared in which Pgpa2–gpa2 was introduced into the FGFP strain so that it expressed Gpa2. Furthermore, Pgit3–git3 was introduced into the FGFP (Gpa2+) strain to create the FGFP (Gpa2+, Git3+) strain.

The fission yeast cells were grown in YES medium (0.5% Bacto yeast extract (Becton, Dickinson and Company) 3% glucose and SP complete supplement (Formedium)) for routine cell growth.

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Received: 09-Oct-2023, Manuscript No: cmb-23-117176; **Editor assigned:** 12-Oct-2023, PreQC No: cmb-23-117176(PQ); **Reviewed:** 26-Oct-2023, QC No: cmb-23-117176; **Revised:** 30-Oct-2023, Manuscript No: cmb-23-117176(R); **Published:** 30-Nov-2023, DOI: 10.4172/1165-158X.1000291

Citation: Sasai K, Hidaka S, Osada T (2023) Construction of a Glucose Biosensor Using the fbp1–GFP Reporter System in the Fission Yeast Schizosaccharomyces pombe. Cell Mol Biol, 69: 291.

Table 1: Strains of fission yeast strains used in this study.

Strains	Genotypes
OSP230	h-, ura4-D18, leu1-32, sxa2Δ, mam2Δ
OSP240	h-, ura4-D18, leu1-32, sxa2Δ, mam2Δ, gpa1Δ
OSP250	h-, ura4-D18, leu1-32, sxa2Δ, mam2Δ, gpa1Δ, git3Δ
OSP260	h-, ura4-D18, leu1-32, sxa2Δ, mam2Δ, gpa1Δ, git3Δ, gpa2Δ
FGFP	h-, ura4-D18, leu1-32, sxa2Δ, mam2Δ, gpa1Δ, git3Δ, gpa2Δ, pSL6Z-Pfbp1-GFP
FGFP (Gpa2+)	h-, ura4-D18, leu1-32, sxa2Δ, mam2Δ, gpa1Δ, git3Δ, gpa2Δ, pSL6Z-Pfbp1-GFP, pSU1Z-Pgpa2-gpa2-TLPI
FGFP (Gpa2+, Git3+)	h-, ura4-D18, leu1-32, sxa2Δ, mam2Δ, gpa1Δ, git3Δ, gpa2Δ, pSL6Z-Pfbp1-GFP, pSU1Z-Pgpa2-gpa2-TLPI, pM3-Pgit3-git3

Construction of plasmids

The design of the reporter plasmid was based on the pSL6Z vector that integrates into the yeast chromosome at a position upstream of the leu1 gene. The major fragment, including the origin of replication, selectable marker, and lipocortin I terminator (TLPI), was amplified from pSL6Z using the primers pSL6Z_F and pSL6Z_R and digested with PstI and SalI. Fragments containing Pfbp1 were amplified from the genomic DNA of S. pombe using the primers Pfbp1_F and Pfbp1_R and digested with PstI and NcoI. The open reading frame (ORF) of GFP was amplified using GFP_F and GFP_R primers from the Monster Green fluorescent protein phMGFP vector (Promega Japan) and digested with NcoI and SalI. The GFP fragments Pfbp1 and ORF were inserted into the PstI–SalI site of the main fragment of pSL6Z. The Pgpa2 and gpa2 genes were inserted into the pSU1Z plasmid to express the gpa2 gene under the control of the gpa2 promoter (Pgpa2). The yeast chromosome was located upstream of the ura4 gene. The major fragment, including the origin of replication, selectable marker, and TLPI, was amplified from the pSU1Z vector using the primers pSU1Z_F and pSU1Z_R. The gpa2 gene was amplified from cDNA resulting from reverse transcription of total RNA from S. pombe using the primers Gpa2_F and Gpa2_R. The gpa2 gene was ligated with the main fragment of pSU1Z. Reverse PCR was performed on the obtained plasmid using the primers Gpa2_F and pSU1Z_R. The resulting fragment was ligated with Pgpa2 amplified from the genomic DNA of S. pombe using the primers pSU1Z–Gpa2_F and pSU1Z–Gpa2_R to generate pSU1Z–Pgpa2–gpa2.

For the Git3, a plasmid was prepared as follows based on the pM3 vector. The major fragment, including the origin of replication, selectable marker, and TLPI, was amplified from pM3 using the primers pM3_F and pM3_R and digested with XbaI and SalI. Pgit3¬–git3 was amplified in the same manner as the gpa2 gene using the primers PGit3_F and Git3_R. These fragments were digested by PstI and SalI and inserted into the PstI¬–SalI site of the main fragment of pM3.

All PCR products used for plasmid construction were prepared using KOD-plus-Neo (TOYOBO Co.) according to the supplier's instructions. All restriction enzymes were purchased from New England Biolabs. The ligation reaction was performed using a ligation convenience kit (Nippon Gene Co.). The sequence of each plasmid was confirmed by nucleotide sequence analysis. Reverse transcription was performed using a Prime Script II first-strand cDNA synthesis kit (Takara Bio). All resulting plasmids were digested with NotI prior to transformation (Table 2).

Transformation

The fission yeast was transformed using a lithium acetate method [26,27]. Transformants were plated onto an SD agar plate (0.17% YNB without ammonium sulfate (MP Biomedical), 5% ammonium sulfate, 2% glucose and 1.6% Bacto agar (Becton, Dickinson and Company)) supplemented with 20 mg/mL uracil, or an SD agar plate supplemented with 30 mg/mL leucine. The plates were incubated at 32°C for 4–6 days, then positive colonies were selected. To check for correct integration, PCR was performed on the extracted DNA using Sapphire Amp fast PCR master mix (Takara Bio).

Table 2: Primers used in this study.

Primers	Nucleotide sequences (from 5' to 3')
pSL6Z_F	AAAGTCGACGCATGCAAGCTTAAATAGGAA
pSL6Z_R	AAACTGCAGGCGCAATTTCAACAATTCCT
Pfbp1_F	AACTGCAGATTCCTACTCGCATCGCATT
Pfbp1_R	AAACCATGGGATGGAGTAAACGAAACCTGAAT
GFP_F	AAACCATGGGCGTGATCAAGCCCG
GFP_R	AAAAAAAAGGTCTCGTCGACTTAGCCGGCCTGGCG
pSU1Z_F	CACAATTCCACACAACATACGAGC
pSU1Z_R	AAAAAGAATTCACCCCGTAATTGATTACTACGCG
Gpa2_F	ATGACGATTTTTAATGGATTATCTGA
Gpa2_R	TTAAAACATTCCCGCTTCTTTC
pSU1Z-Gpa2_F	CGAAAACATGCTTTGCTTCA
pSU1Z-Gpa2_R	TTTTTTTTAATCCACTACTGCAGAATATAA
pM3_F	AAAGTCGACGGCATGCAAGCTTAAATAGGA
pM3_R	AAAAATCTAGAAACCTGATGCTGATGTTTCG
PGit3_F	AACTGCAGCCTCGTTTGTCTGTGCAAAA
Git3_R	AAAGGTCTCGTCGACCTATTTTTCCTCACCAAATTTACCCC

Gene disruption

The gene disruption of gpa1, git3, and gpa2 was performed by a standard homologous recombination method. The details of plasmid construction for gene disruption have been described previously [28]. Briefly, about 1000 bp of 5′ and 3′ flanking sequences of the target gene were used as the chromosomal integration regions. To delete the ura4 selection marker, the ura4 gene was sandwiched with about 200 bp of 3′ flanking sequence of the target gene. For the negative selection of the ura4-expressing cells, the cells were plated onto YES-FOA plates (0.5% Bacto yeast extract, 3% glucose and SP complete supplement, 1.5% Bacto agar, and 0.4% 5-fluoroorotic acid). The resultant ura4-expressing cells were used for subsequent gene recombination.

Glucose signaling assay

The cells were grown in YES medium at 32°C for 24–36 hours and inoculated into 5 mL of fresh YES medium. Then, the cells were grown at 32°C for 24 hours and collected. After washing twice with sterile water, cells were cultured in DOB medium containing each concentration of glucose and 3% glycerol (0.17% YNB without ammonium sulfate (MP Biomedical), 5% ammonium sulfate, 3% glycerol). GFP has peak fluorescence at a wavelength of 515 nm. In this study, the signal intensity of each strain obtained in each assay was calculated as the height of the fluorescence intensity peak of the expressed GFP. Fluorescence intensity of GFP was measured by a Hitachi F-2700 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan). The obtained signal intensity was converted into a numerical value relative to the OD600 = 1. The peak height was obtained as

follows: The fluorescence intensity was measured with the excitation wavelength of 480 nm, and the straight line connecting the values at 505 nm and 535 nm of the obtained fluorescence spectrum was used as the baseline. Then, the baseline value at 515 nm was subtracted from the 515 nm value, which is the peak of the fluorescence. The results obtained were analyzed using the t-test. A p value less than 0.05 was considered statistically significant.

Results and Discussion

To develop a yeast-based glucose signaling assay, we constructed a new reporter that caused yeast cells express GFP in the absence of glucose. The fbp1 gene encodes the gluconeogenic enzyme fructose-1,6-bisphosphatase, which is transcriptionally repressed by glucose [18]. In previous studies, we isolated a yeast strain in which fbp1 is constantly transcribed as a glucose-insensitive transcription mutant, and discovered several factors involved in the glucose signaling pathway. In this study, we constructed an fbp1–GFP fusion reporter to measure glucose concentrations (Figure 1). In glucose-rich conditions, cAMP produced by activated Cyr1 activates Pka1 and suppresses the transcriptional activity of Rst2 on the fbp1 gene, so the Fbp1-GFP fusion protein is not expressed. In conditions of glucose starvation, cAMP is not produced, so Pka1 is not activated and Rst2 is not phosphorylated, resulting in transcription of GFP.

To evaluate the utility of the fbp1–GFP fusion reporter as a glucose biosensor, a git3Δgpa2Δ double deletion strain (FGFP), a gpa2+ strain (FGFP (Gpa2+)) and a git3+ gpa2+ strain (FGFP (Gpa2+, Git3+)) were constructed. The GFP expression level was assayed for all strains grown in the presence or absence of glucose as shown in Figure 2. All strains grown in 0 mM glucose showed high levels of GFP expression. In the presence of 100 mM glucose, the FGFP (Gpa2+, Git3+) strain did not express GFP. In addition, the FGFP and FGFP (Gpa2+) strains expressed GFP and their fluorescence intensities were lower than that of the FGFP (Gpa2+, Git3+) strain.

The fbp1 promoter contains two upstream activation sites (UAS), UAS1 and UAS2, in the required for activation of fbp1 transcription (Figure 3) [29]. Glucose starvation stimulates the stress-response signaling pathway, and the signal is mediated by the transcription

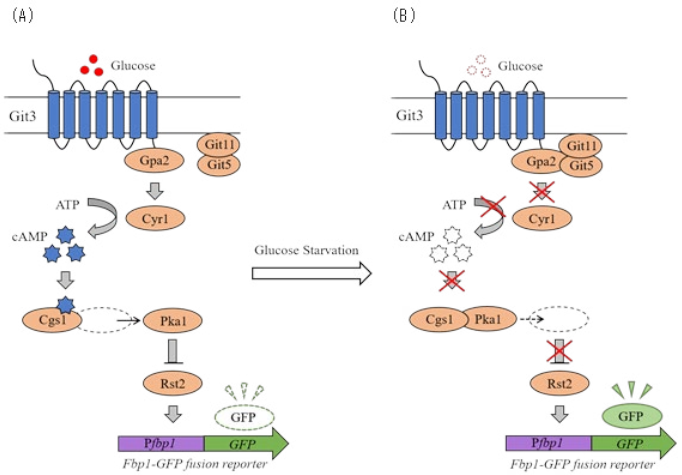

Figure 1: The constructed assay system was based on the glucose signaling pathway. The transcriptional activity of the fbp1–GFP fusion under glucose-rich and glucose-starvation conditions (A) Under glucose-rich conditions, Gpa2 is activated, which activates Cyr1 to produce cAMP. Pka1 phosphorylates Rst2 and suppresses the expression of GFP. (B) Under glucose-starvation conditions, cAMP synthesis is suppressed, so Pka1 does not phosphorylate Rst2, and GFP expression is induced. (The fbp1 promoter is written as Pfbp1).

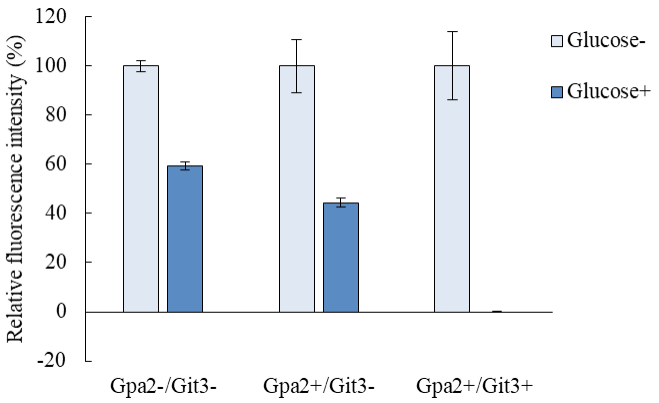

Figure 2: Changes in fluorescence intensity due to the expression of Gpa2 and Git3. FGFP, FGFP (Gpa2+), and FGFP (Gpa2+, Git3+) strains were used in this study. Glucose was added to DOB medium containing 3% glycerol at a final concentration of 100 mM, and the fluorescence intensity was measured after 24 hours. The change in fluorescence due to the addition of glucose is represented by taking the average value of the fluorescence intensity of a sample without glucose as 100%.

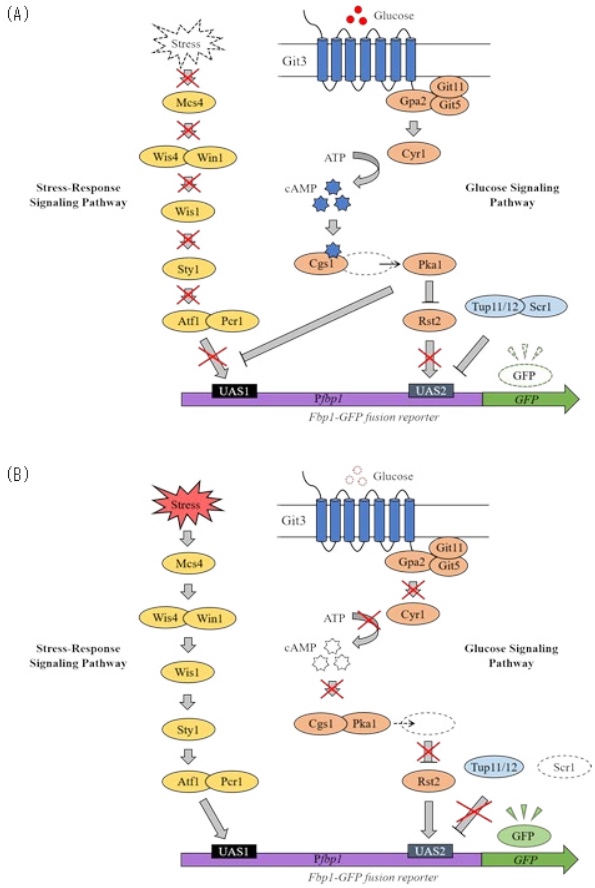

Figure 3: The stress-response signaling pathway. There are two upstream activation sites (UAS), UAS1 and UAS2, on the fbp1 promoter. UAS1 and UAS2 are regulated through both the glucose signaling pathway and the stress-response signaling pathway. (A) In the glucose-rich state, activation of the fbp1 promoter is not initiated in UAS1 because the stress-response pathway is not activated. Similarly, transcriptional activation of the fbp1 promoter is not initiated in UAS2 because Tup11/12, and Scr1 bind to UAS2 and inhibit transcription. (B) In glucose-starvation conditions, the stress-response signaling pathway is activated, the transcription factor Atf1–Pcr1 binds to UAS1, and transcription of the fbp1 promoter is activated. In addition, the transcription factor Rst2 is not phosphorylated by Pka1, and so competes with Tup11/12 to bind UAS2. As a result, transcriptional activity of the fbp1 promoter occurs and GFP is expressed.

factor dimer Atf1¬–Pcr1. Atf1–Pcr1 binds to UAS1 and de-represses fbp1 transcription. In glucose-rich conditions, Pka1 activation in the glucose signaling pathway inhibits binding of Atf1–Pcr1 to UAS1. At UAS2, the transcriptional co-repressor Tup11/12 and the transcription factor Scr1 exported from the nucleus repress fbp1 transcription under glucose-rich conditions. During glucose starvation, Scr1 is not exported and Rst2, which antagonizes the function of Tup11/12, de-represses fbp1 transcription [30,31]. Therefore, the high expression of GFP is likely due to inhibition of the glucose signaling pathway as well as activation of the stress-response signaling pathway by glucose starvation. In the presence of 100 mM glucose, a glucose-rich condition, the FGFP (Gpa2+, Git3) strain did not express GFP due to activation of the glucose signaling pathway and inhibition of the stress-response signaling pathway. In addition, it is probable that the lower fluorescence intensity of FGFP and FGFP (Gpa2+) strains, which lack the glucose signaling pathway, in the presence of glucose is due to the repression of stress-response signaling under glucose-rich conditions and the repression of glucose signaling by knockout of Gpa2 and Git3.

Figure 4 shows the time course of the glucose assay under glucose starvation and glucose-rich conditions. In the absence of glucose, the fluorescence intensity of FGFP (Gpa2+, Git3+) strains gradually increased. The fluorescence intensity doubled in 12 hours and increased about 6-fold in 24 hours. In the presence of 100 mM glucose, the fluorescence intensity of the strains halved in about 6 hours and was absent by 12 hours. Under glucose starvation, the input of stress signals to the fbp1 promoter promoted GFP expression and increased fluorescence intensity over time. Under glucose-rich conditions, stress signals due to glucose starvation were suppressed and GFP expression was suppressed by activation of the glucose signaling pathway, resulting in complete disappearance of fluorescence.

Figure 5 shows the concentration dependency of the glucose assay. As the glucose concentration increases, the fluorescence intensity of the strains decreased in a concentration-dependent manner. The fluorescence intensities of the strains at 1 μM glucose were lower than those of the strains in the absence of glucose, the fluorescence intensity

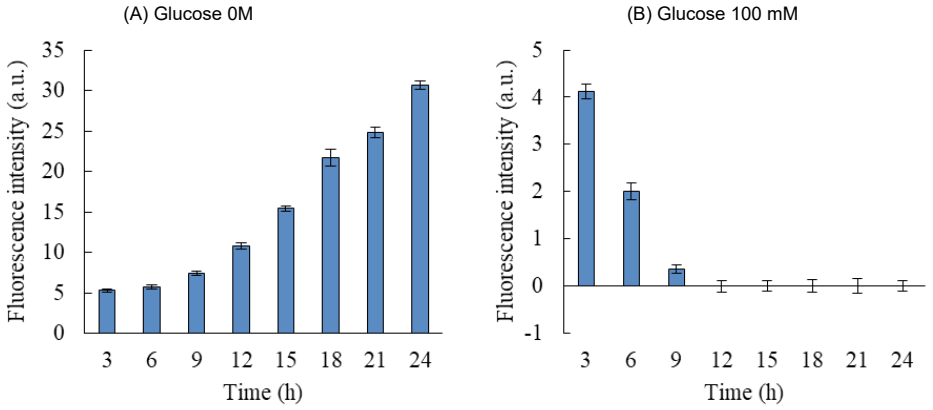

Figure 4: Changes in glucose concentration and fluorescence intensity over time. Using the FGFP (+Gpa2, +Git3) strain, glucose was added to DOB (3% glycerol) medium at a final concentration of 0 M (A) and 100 mM (B). After the addition of glucose, the fluorescence intensity was measured every 3 hours for 24 hours.

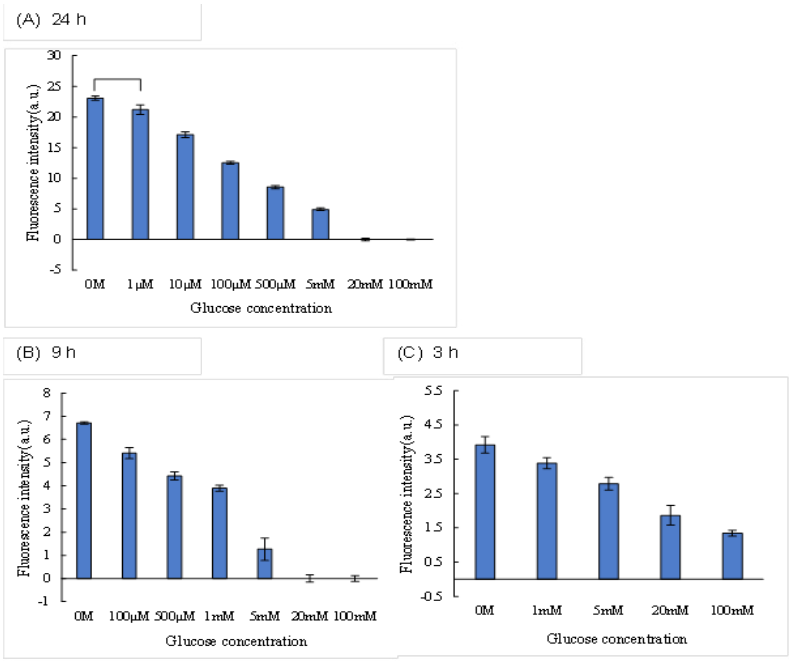

Figure 5: Fluorescence intensity depends on the glucose concentration. Using the FGFP (+Gpa2, +Git3) strain, the fluorescence intensity was measured 24 hours (A), 9 hours (B) and 3 hours (C) after addition of glucose. Glucose was added to DOB medium containing 3% glycerol at each concentration. The results obtained were analyzed using the t-test. A p value less than 0.05 was considered statistically significant. *not significant. **$p < 0.05$.

at 100 μM glucose was approximately half that at 1 μM glucose, and fluorescence was absent at 20 mM glucose. However, the fluorescence decrease at 1 μM was not significantly different. Therefore, this significant decrease in fluorescence intensity at 10 μM glucose indicated that the reporter system can measure a glucose concentration of 10 μM as in Figure 4(A). In shorter time of incubation, 100 μM and 5 mM glucose were detected at 9 hours (B) and 3 hours (C), respectively.

Conclusions

To develop a glucose biosensor, we constructed a hybrid fbp1–GFP gene fusion reporter for monitoring the expression level of GFP in response to glucose. The method can measure glucose concentrations of about 10 μM, which is sufficient to determine a range of glucose levels in human blood, saliva, and tears. The yeast strains developed in this study as a glucose biosensor may also be used as a platform for analyzing the activity of heterologous GPCRs; particularly orphan GPCRs, in future studies.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contribution

Kanako Sasai: Methodology, Validation, Formal analysis, Investigation, Writing- Original draft preparation. Sho Hidaka: Methodology. Toshiya Osada: Writing- Review & Editing, Supervision.

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