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Recovery of active recombinant EGFP from the excrement of transgenic mice: A possible source of recombinant protein

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ABSTRACT

Transgenic animals provide attractive systems for the production of valuable recombinant proteins. Previous studies indicate that milk is a suitable source of secreted recombinant proteins. In the current study, we examine whether excrement can be another source of recombinant proteins by using transgenic mice ubiquitously-expressing green fluorescent protein (GFP) as a model. We found that the surface of excrement from GFP-transgenic mice was fluorescent, and the supernatant after centrifugation of an excrement suspension was rich in undegraded, actively fluorescing GFP. GFP was successfully purified from stool as a fluorescent 27 kDa protein by using a common procedure. Finally, we observed that the fluorescence of digested materials began in the ileum and persisted throughout the remainder of the digestive tract. Our results demonstrate that excrement, which is produced daily regardless of the sex or age of the animal, may be another feasible source of recombinant proteins. The preparation method is simple, economical, and noninvasive.

1. Introduction

Many systems are available for producing recombinant proteins in a variety of living creatures, including *Escherichia coli*, *Saccharomyces cerevisiae*, and mammals [1]. However, each system has its own disadvantages [2]. Although *E. coli* provides a powerful system that can even produce human recombinant proteins [3], it often fails to produce biologically active proteins because of the formation of aggregates, lack of preprotein cleavage, and loss of post-translation modifications such as glycosylation, carboxylation, and phosphorylation. Therefore, mammalian cells are more suitable for producing functional proteins because they are often made in a properly folded and glycosylated form, can undergo fatty acid chain addition, and can be phosphorylated on tyrosine, threonine, and serine residues [2].

In terms of posttranslation modifications and appropriate protein folding for biological activity, transgenic animals provide an attractive and powerful system for producing valuable recombinant proteins relevant to human healthcare [2,4]. Transgenic animals carry exogenous genes integrated into their genome, thus allowing for the inheritance and expression of these genes in their offspring. Transgenic mice, rats, goats, cows, pigs, rabbits, and sheep are currently being developed, with recombinant proteins produced in milk, blood, urine, and seminal plasma [4]. The expression of recombinant proteins in milk is currently one of the most developed systems for recombinant protein production in transgenic organisms [5–7]. However, the production of recombinant proteins in mammary glands is restricted to sex, species, and protein expression time period. Protein recovery from urine is difficult, and recombinant protein expression in both milk and urine is restricted to those that are secreted.

Stool contains a variety of proteins, as well as DNA and RNA, from the host, microflora, and diet. For example, human stool

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samples (1 g) contain a mean of 100 μg DNA; human DNA comprises 0.9 mg/g of the total fecal DNA [8]. Therefore, stool provides a noninvasive source of information regarding the health status of the host and the microbiota. Stool is produced daily regardless of the sex and age of the animal and can be collected easily. Furthermore, recombinant protein in stool would be easier to purify than that in tissues and organs. The use of stool as a source of recombinant protein could be beneficial in terms of cost and efficiency.

The green fluorescence protein (GFP) was originally isolated from the luminescent jellyfish *Aequorea victoria* [9] and forms an intrinsic chromophore by the cyclization and oxidation of an internal tripeptide motif (Ser-Tyr-Gly) [10]. The expression of GFP in heterologous systems has been widely applied since its first success in *Caenorhabditis elegans* [11] and provides a powerful tool as a dynamic marker in live cells. Furthermore, various mutant GFPs with modified fluorescence spectra and increased extinction coefficients have been developed, including enhanced GFP (EGFP) [12]. GFP remains remarkably stable in the presence of many denaturants and proteases and over a broad range of pH and temperatures [13,14].

This study uses GFP as a traceable, recombinant protein expressed in transgenic mice to examine the feasibility of using stool as a new source of recombinant proteins.

2. Materials and methods

2.1. Animals

GFP-expressing transgenic ICR mice (GFP mice) previously generated using pCX-EGFP (an EGFP-containing vector) [15] were used in this study. ICR mice were obtained at age 8–12 weeks from Kiwa Laboratory Animals (Wakayama, Japan). All animal procedures conformed to the Guidelines of Kindai University for the Care and Use of Laboratory Animals.

2.2. Microscopic observation of GFP fluorescence

The microscopic documentation of fresh excrement samples without fixation was performed using an SZX 16 fluorescent stereomicroscope equipped with a DXC-S500/OL camera system (Olympus Optical Co. Ltd., Tokyo, Japan).

For histological analysis, excrement samples from GFP mice were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (136.89 mM NaCl, 2.69 mM KCl, 3.21 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 (pH 7.0)) overnight at 4 °C and washed in PBS containing sucrose (6.8%). After washing in PBS for 4 h, the tissues were placed in acetone for 1 h and then embedded in glycol methacrylate (Technovit 8100; Kulzer, Hanau, Germany). Serial cross sections (thickness, 5 μm) were cut and embedded in Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). These sections were examined under an Olympus BX51 fluorescence microscope equipped with a DP70 Digital Camera (Olympus).

2.3. Fluorescence assay

Fresh excrement and materials from the digestive tract were immediately suspended in PBS to a final concentration of 10% wet excrement weight/volume. For fluorescence assays of supernatants and precipitates, this suspension was centrifuged for 5 min at 10,000 × g. The precipitate was resuspended in PBS. Following a 10-fold dilution of each sample with PBS, 100 μL of the suspension was transferred into 384-well FIA Black Plates (781086; Greiner Bio-One, Kremsmuenster, Austria). The fluorescence intensity of GFP was measured using EnVision 2103 Multilabel Readers (Perkin Elmer Life Sciences, Waltham, MA, USA) with excitation at 485 nm and emission at 535 nm.

2.4. SDS-PAGE and Western blot analysis

Excrement suspended in PBS as described above was diluted 200-fold in PBS, mixed with an equal volume of SDS sample buffer (β-ME Sample Treatment for Tris SDS; Cosmo Bio, Tokyo, Japan), and then solubilized. A total of 10 μL of each sample was then boiled for 5 min at 95 °C, resolved by SDS-PAGE on a 12.5% gel, and visualized using SimplyBlue (LC6065; Thermo Fisher Scientific, Scotts Valley, CA, USA). A ladder of Precision Plus protein standards (BioRad, Hercules, CA, USA) was used as a molecular mass marker. For Western blot analysis, proteins were transferred to a PVDF membrane (GE healthcare, Little Chalfont, UK), followed by blocking with 40 g/L Bock ACE (Dainippon Sumitomo Pharma Co., Ltd.). Mouse anti-GFP monoclonal antibody against amino acids 1–238 (full-length GFP) (B-2, sc-9996; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:1000 in PBS containing 0.2% Tween 20 (0.2% PBS-T) with 4 g/L Bock ACE. A sheep anti-Mouse IgG antibody conjugated to HRP (Millipore, Burlington, MA, USA) was diluted 1:10,000 in 0.2% PBS-T with 4 g/L Bock ACE. Blots were imaged on an X-ray film by chemiluminescence by using the ECL Advance Western Blotting Detection Kit (RPN2135; GE healthcare). A band at 27 kDa corresponding to the predicted molecular weight of GFP was detected only in samples from mice carrying the GFP transgene.

2.5. Purification of GFP

Two grams of excrement was suspended in 50 mL of 50 mM phosphate buffer (pH 7.0) including NaCl, 0.2% glycerol, 2 mM ascorbic acid, 2 mM phenylmethylsulfonyl fluoride, and 5 mM ethylenediaminetetraacetic acid and then stirred at 4 °C for 1 h. This suspension was centrifuged for 30 min at 10,000 × g. The supernatant was passed through a 0.2 μm Minisart syringe filter (Sartorius Stedim Biotech, Göttingen, Germany) to remove trace amounts of precipitates. Ammonium sulfate was added slowly to the supernatant to 20% saturation. This suspension was centrifuged for 30 min at 10,000 × g. To precipitate the target protein, ammonium sulfate was slowly added to the supernatant up to 80% saturation. The precipitate was collected by centrifugation at 10,000 × g for 30 min and then dissolved in a small volume of 50 mM phosphate buffer (pH 7.0) including 1 M $(NH_4)_2SO_4$. Hydrophobic interaction chromatography was used to separate the recombinant GFP from contaminant proteins. The resultant clear solution was applied to a Toyopearl Phenyl-650C column (TOSOH, Tokyo, Japan) equilibrated with the same phosphate buffer. The column was eluted with approximately five bed volumes of reverse salt steps of 0.75, 0.5, 0.25, and 0 M $(NH_4)_2SO_4$. To identify the GFP fraction, each fraction was diluted 10 times with PBS, and the specific fluorescence was measured.

3. Results

3.1. Microscopic observation of stool derived from GFP mice

We first microscopically observed GFP fluorescence in stool derived from homozygous (*Gfp/Gfp*) and heterozygous (*Gfp/−*) GFP mice. This stool exhibited uniform green fluorescence, with the exception of hair and red blood cells [15]. Although stool from GFP mice was indistinguishable from that of control wild type ICR mice (*−/−*) without excitation light, they became distinguishable by green fluorescence under excitation light, thus suggesting that the