

Demonstration of protein absorption in the intestinal epithelium of fish and mice by laser scanning confocal microscopy

Wan-fu Yue¹, Fang Zhou¹, Firdose Ahmad Malik¹, Hua-jun Yang¹, Xing-hua Li¹, Bhaskar Roy¹, Srimivasa Reddy² and Yun-gen Miao^{1,*}

¹Key Laboratory of Animal Epidemic Etiology and Immunological Prevention of Ministry of Agriculture, College of Animal Sciences, Zhejiang University, Hangzhou 310029, P.R. China

²Department of Sericulture, University of Mysore, Karnataka 570006, India

*Corresponding author
e-mail: miaoyg@zju.edu.cn

Abstract

Selective permeability for small proteins and oligopeptides occurs in the intestinal epithelium of many animal species and humans. Whole proteins are sometimes endocytosed and undergo partial hydrolysis in intestinal epithelial cells with the probable release of essential oligopeptides into the bloodstream. Increased permeability to certain proteins can cause asthma and other metabolic disorders. Permeable proteins have also been successfully used to deliver vaccines or drugs via oral consumption. Protein absorption has been inferred in many cases and demonstrated in some cases by histochemical, tracer, and analytical techniques. However, the nature and importance of protein absorption remains largely unknown. Here, we demonstrate the movement of two luminal proteins (GFP: 26 kDa and OFP: 23 kDa) across the intestinal epithelium of fish and mice using laser scanning confocal microscopy. The results provide evidence that small proteins can be taken up intact by intestinal epithelial cells, even though large proteins are digested to single amino acids or protein fragments before they are absorbed. Our results suggest that it is possible to orally administer some small proteinous medicines for therapeutic purposes.

Keywords: *Bombyx mori* L.; *Clarias leather*; intestinal epithelial cells; mice; OFP and GFP; silkworm.

Introduction

The intestinal epithelium is a selective barrier between the intestinal lumen and systemic circulation. Recent studies have shown that the human digestive tract preferentially absorbs protein fragments in the form of di- and tripeptides. In fact, more than 70% of the amino acids that appear in the blood after ingestion are di- and tripeptides with potent bio-

logical activities, influencing everything from hormones to immune and nervous system function. These findings are inconsistent with the outdated belief that all proteins are absorbed in the form of single amino acids. One factor emphasized in these studies is that the permeability of the gut is a major factor in many illnesses. If the gut is not properly nourished with protein then it will become more permeable and this can lead to problems such as allergies and immune system compromise as well as hormonal imbalances. However, in healthy adults, it is well known that the permeability of an intestinal solute through the intercellular junction (paracellular pathway) is highly dependent on its size (Donovan et al., 1990; He et al., 1998). Molecules that hardly interact with intestinal tissue might exhibit a simple correlation between their molecular size and rate and extent of absorption. Therefore, the intestinal epithelium of healthy adults is generally considered to be virtually impermeable to macromolecules. In addition to the mechanical barrier presented by the tissue, the enzymatic barrier, i.e., rapid and extensive degradation of proteins by digestive enzymes, highly restricts the entry of intact, undigested proteins into the body. However, some studies support the idea that there is a small, but significant, degree of transport of biologically and/or antigenically active peptides and proteins through the epithelial cells of the intestines (Beglinger et al., 1992; Lovegrove et al., 1993; Castell et al., 1997).

Protein absorption is a common condition but its pathophysiology remains poorly understood. Many food allergy patients give a history of food intolerance, but data from dietary elimination and rechallenge studies are inconclusive. Multiple etiopathological mechanisms have been postulated. The gut has an extensive immune system but current understanding of processing of food antigens in health and disease is limited.

In the present study, we selected several species such as the catfish *Clarias leather* (carnivorous) and mouse (omnivorous) to examine protein absorption by animal intestinal epithelial cells using laser scanning confocal microscopy (LSCM). The aim of this study was to determine whether proteins escaping intracellular digestion pass through the epithelial cells of the intestine.

Results

LSCM images of blood smears of OFP-fed mice

Prior to treatment, the mice were fasted overnight. The mice received hemolymph of silkworm containing OFP-expressed or an equivalent amount of the normal worm hemolymph

orally, respectively. After 15 days of oral feeding, the blood sample was drawn from the mice and the blood smear was viewed through confocal laser scanning microscopy (CLSM).

Orange fluorescence was clearly observed under UV light in the blood smear of mice fed with hemolymph containing expressed OFP (Figure 1, A-1, A-2), whereas no such fluorescence was observed in control mice which were fed with the normal worm hemolymph (Figure 1, B-1, B-2).

LSCM images of intestinal epithelial cells of OFP-fed mice

The mice were sacrificed and the intestines were dissected from control and experimental groups in cold physiological saline solution after 15 days of oral feeding with silkworm hemolymph containing OFP. Figure 2 shows the epithelial cell images observed by CLSM.

CLSM revealed orange fluorescence along the intestinal epithelial cell (iec, Figure 2A) in the small intestine of treated mice. The magnified image in Figure 2B shows orange fluorescence in the villi and basement membrane and in the cytoplasm of absorbing cells. No OFP was observed in the intestinal epithelial cells of control mice (Figure 2C).

LSCM images of blood smears of OFP-fed fish

The *Clarias leather* fish were treated by feeding once per day with 0.5 ml of silkworm hemolymph containing OFP. Blood was collected after 20 days of oral feeding and examined under a UV microscope.

OFP was observed in the blood smear of fish fed on a daily dose of OFP for 20 days (Figure 3A) and not in the control fish (Figure 3B).

LSCM images of intestinal epithelial cells of OFP-fed fish

After 20 days of oral feeding with OFP-expressed silkworm hemolymph, the *C. leather* fish were sacrificed and the intestines were removed from control and experimental fish in cold physiological saline solution.

OFP was clearly detected all along the villi and basement membrane of the lining epithelial cells of the intestine (Figure 4A). However, no orange fluorescence was detected in the control fish (Figure 4B).

LSCM images of blood smears of GFP-fed fish

In replacement of the OFP experiment, another small fluorescence protein, GFP, was used to investigate further the absorption of proteins.

C. leather fish were fed on a daily dose of hemolymph containing GFP for 20 days. Green fluorescence was observed in the blood smear from GFP-treated fish under UV (Figure 5, A-2), but not control fish (Figure 5, B-2).

LSCM images of intestinal epithelial cells of GFP-fed fish

When the excitation and emission were set at 488 and 504 nm and the collection wavelengths at 510 to 545 nm,

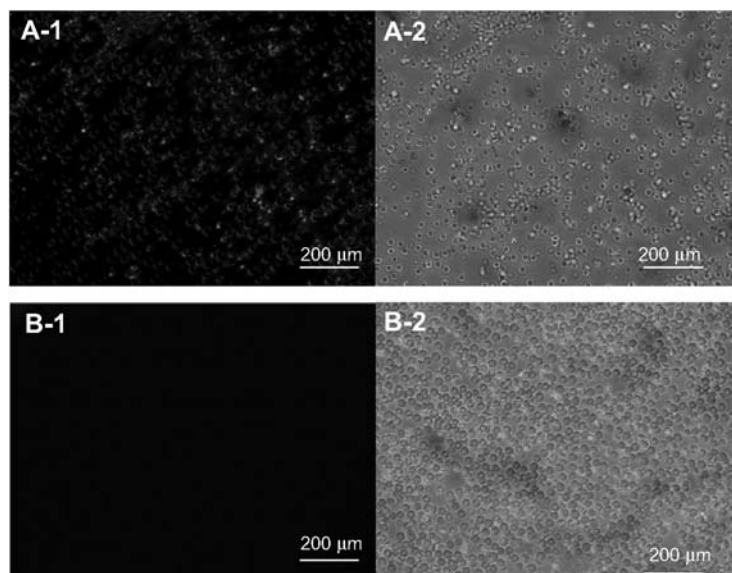


Figure 1 Image of mice blood under UV and normal light after OFP oral treatment.

(A-1) Image of treated mice blood under UV (original magnification 40×); (A-2) treated mice blood under normal light; (B-1) controlled mice blood under UV (40×); (B-2) controlled mice blood under normal light (100×). The mice were randomly divided into three groups of 10–15 animals in each group. The 1st and 2nd groups orally received the hemolymph of silkworm containing OFP-expressed three times a day and the 3rd group (control) of mice received an equivalent amount of the normal silkworm hemolymph.

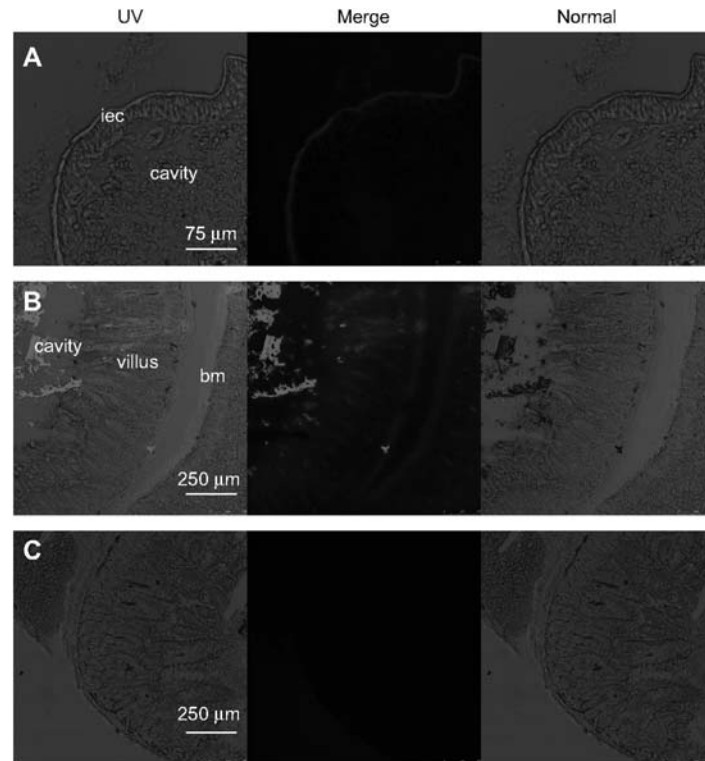


Figure 2 LSCM images of intestinal epithelial of mice cells after OFP oral treatment.

The OFP was clearly detected throughout the intestinal epithelial cell (iec) in panel (A). Panel (B) shows an enlarged LSCM image, the orange fluorescence (light gray) is present in the villus and base membrane (bm). In contrast, no OFP was observed in intestinal epithelial cells from control mice (C). Left, image under UV; middle, merged; right, image under normal light.

green fluorescence was clearly detected throughout the intestinal epithelial cell (iec) and cavity (Figure 6A). The intestinal epithelial cells of control *C. leather* showed no GFP fluorescence (Figure 6B).

Discussion

The mechanisms of protein digestion and absorption have been studied with increasing intensity and a large book could be written on this subject alone. Despite this volume of research, it has recently become clear that our knowledge of protein digestion and absorption is still far from complete,

because until very recently almost all investigations have been focused on the intestinal transport of amino acids.

Protein digestion is initiated by gastric proteinases (pepsins) with pH optima on the acidic side of neutrality and two or more such enzymes are distinguishable in humans and other animals (Lovegrove et al., 1993). Although gastric digestion of protein is rather extensive in the mechanical sense, it is unlikely that it proceeds *in vivo* beyond the stage of producing a mixture of large polypeptides and unaltered protein. The release of free amino acids could well be negligible. Once proteins and the products of peptic digestion have left the stomach and are exposed to the proteolytic enzymes of the pancreas, chemical breakdown is extensive

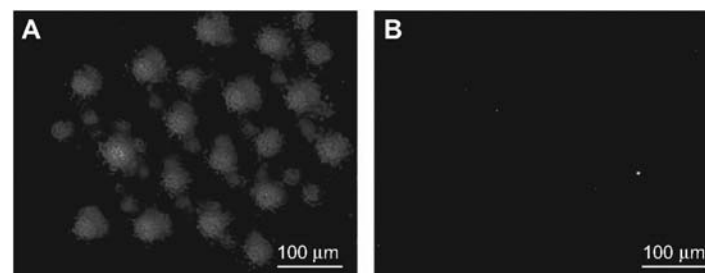


Figure 3 Image of *Clarias leather* blood under UV after OFP oral treatment.

(A) OFP-treated *C. leather* blood; (B) *C. leather* control blood. The original magnification was 100 $\times$.

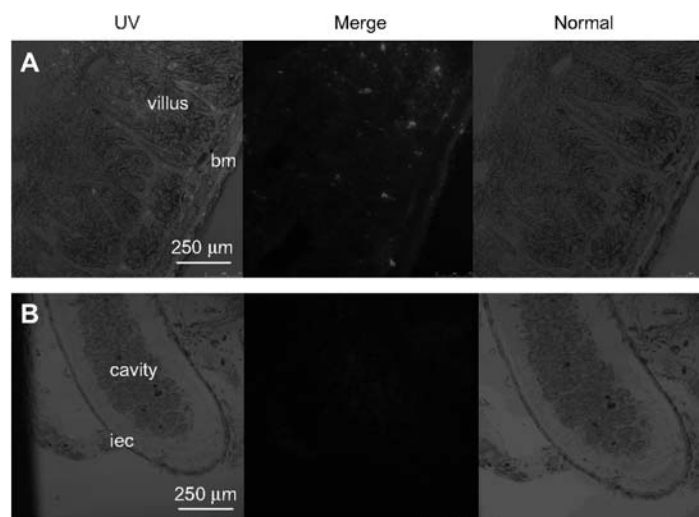


Figure 4 LSCM images of *C. leather* intestinal epithelial cells after OFP oral treatment.

The OFP (regions of light gray) is clearly detected throughout the villus and basement membrane in the intestinal epithelial cells (A), whereas no orange fluorescence was detected in *C. leather* intestinal epithelial control cells (B). Left, image under UV; middle, merged; right, image under normal light.

and extremely rapid. The pancreatic phase of protein digestion is important.

In newborn animals, there is extensive absorption of whole proteins from milk, including maternal antibodies, which occurs by the process of pinocytosis (Taylor, 1968). However, it is still thought that whole proteins are not absorbed in the adult animal except on a very small scale. The occurrence of this in humans is suggested by allergic reactions, which are experienced by some individuals in response to certain foodstuffs. It has gradually become accepted that proteins are completely hydrolyzed to amino acids in the intestinal lumen before absorption, and then absorbed by diffusion.

However, in 1959, Newey and Smyth showed that the dipeptide glycylglycine was taken up intact from the intestinal lumen and hydrolyzed by the mucosal cells (Clark, 1965). This observation initiated the complete breakdown of the hypothesis of intralumen hydrolysis to amino acids. It is presently thought that there are at least two modes of absorption of protein digestion products: (1) absorption of free amino acids and (2) intestinal mucosal uptake of oligopeptides, with hydrolysis to amino acids by the peptidases of the mucosal cells.

In adults too, small quantities of proteins or fragments of potentially antigenic proteins pass from the intestinal lumen

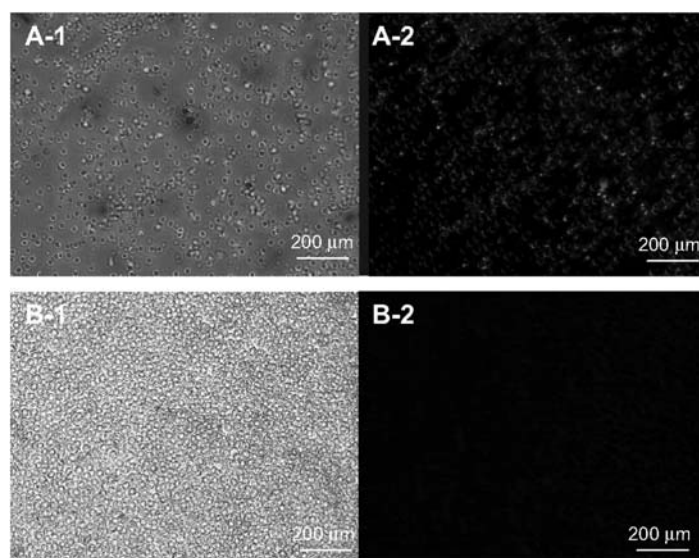


Figure 5 Image of *C. leather* blood under UV and normal light after GFP oral treatment.

(A-1) Blood from GFP-treated *C. leather* animals under normal light; (A-2) blood from GFP-treated *C. leather* under UV; (B-1) blood from control *C. leather* under normal light; (B-2) blood from control *C. leather* under UV. The original magnification was 40×.

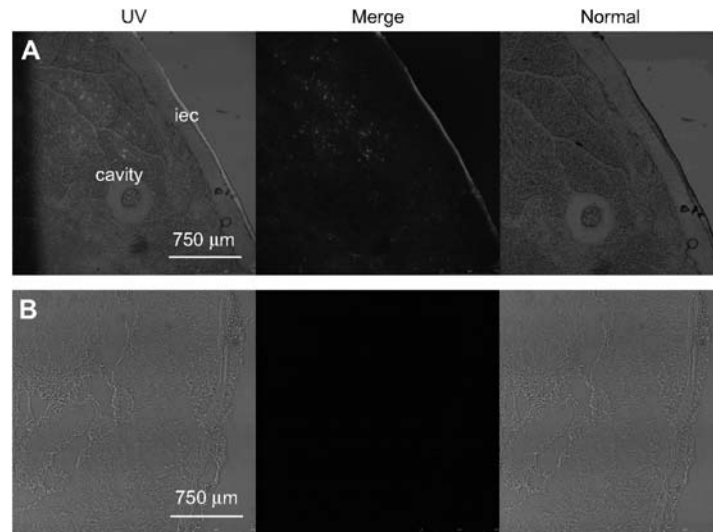


Figure 6 LSCM images of *C. leather* intestinal epithelial cells after GFP oral treatment.

(A) Green fluorescence (light gray) was clearly detected throughout the intestinal epithelial cell (iec) and cavity; panel (B) shows no GFP fluorescence in control *C. leather* intestinal epithelial cells. Left, image under UV; middle, merged; right, image under normal light.

into the lymph and the blood (Newey and Smyth, 1959). At low luminal concentrations the passage of proteins is across specialized cells, the M cells associated with Peyer's patches (Walker, 1980; Bockman et al., 1983), but at higher concentrations, it occurs across all the absorbing cells (Walker, 1980; Bye et al., 1984) and eventually comes in contact with the lymphoid cells of the lamina propria or passes into the blood. According to Jeurissen et al. (1985), particulate protein fractions pass through the M cells and are the origin of local IgA immune responses, whereas soluble protein material passes through the absorbing cells and are the origin of systemic IgG and IgM responses.

From a classical viewpoint of protein absorption, for some medical proteins, vaccine proteins and nutritive proteins, their absorption mechanism remains unclear when they are received orally. In fact, it is easy to administrate these proteins through injection rather than injection, and it is not necessary to purify these proteins when making oral feeding.

Intestinal epithelial cells are mainly charged with digestion and absorption both in mammalian and non-mammalian species. In our previous reports, we used a practical BmNPV bacmid system to express the Mn-SOD enzyme protein in silkworm larvae by the recombinant bacmid baculoviruses (Yue et al., 2006). The availability of large quantities of SOD that the silkworm provides should greatly facilitate future research and testing of this protein for potential application in medicine. We have also investigated the effect of silkworm powder containing SOD on the antioxidation and the immune system of the mouse, focusing on hemolysin response, hemagglutination against SRBC, PFC assay and the dose-dependent increase of phagocytic activity. All treated mice showed significant enhancement of immunity (Yue et al., 2007, 2008).

The SOD is an 18.0-kDa protein. When it is orally administered in mice, can it be absorbed by intestinal epithelial cells? To trace the protein pathway, in the present study, we

used mice and catfish (*Clarias leather*) to investigate the absorption of small molecular size proteins, such as OFP and GFP, which are approximately 23–26 kDa. The results of CLSM showed that such small size proteins can be taken in by the intestinal epithelial cells, and can pass transcellularly or paracellularly through the intestinal epithelium and can be absorbed into circulation. Altered properties of the intestinal epithelial cells would explain the difference in permeability. Such changes lead to the increased nonspecific adsorption of proteins to the cell (Sanderson and Walker, 1993). This implies that some proteinous medicines could possibly be taken in orally. Here, we discuss absorption of small proteins (approx. 23–26 kDa). The absorption of large GFP fusion proteins should be studied further in the future.

In conclusion, most proteins, especially large proteins, probably need to be digested into single amino acids or protein fragments (dipeptide or tripeptide) before being absorbed. However, our observations suggest that some small proteins can be absorbed directly after oral feeding. These findings provide useful information about the intestinal absorption of proteins of immunological and/or pharmacological importance in normal, healthy subjects. This suggests that small size proteins such as vaccines and/or medicines are possible to use orally, not through injection of purified products.

Materials and methods

Expression of OFP

rBacmid/BmNPV/OFP was constructed according to our previous method (Liu et al., 2009). In total, 6 μ g of purified rBacmid/BmNPV/OFP DNA was suspended in 300 μ l of unsupplemented Grace's Insect Cell Medium. Then, 30 μ l aliquot was diluted with 270 μ l of the medium. The contents were thoroughly mixed and incubated for 45 min at room temperature.

Then, 10 μ l of the diluted medium containing rBacmid/BmNPV/OFP was injected into the dorsal aorta of fifth instar silkworms. The development of orange fluorescent protein was monitored everyday postinfection in the dark with an UV illuminator. The hemolymph was drawn on days 7 or 8, centrifuged at 500 *g* for 5 min, and the clear supernatant was stored for *per os* application to fish and mice.

Expression of GFP

BmN cells in a T25 flask were transfected with Bacmid DNA of BmNPV-Polyhedrin-mel-GFP at a volume of 200 μ l and a ratio of 3:2 (μ l FuGene to μ g Bacmid). Cells were observed under UV microscope after 5 and 6 days post-transfection for EGFP expression.

Silkworm larvae at the beginning of the fifth instar were infected with the same transfection solution (recombinant virus) at a volume of 5 μ l per larvae. Most of the larvae expressed GFP after 4 days postinfection. The hemolymph was drawn from the larvae, centrifuged at 500 *g* for 5 min, and the clear supernatant was stored for *per os* application to fish and mice.

Mice and breeding conditions

A total of 75 male ICR mice from the Animal Experiment Center of Medical College, Zhejiang University, China, in the weight range of 18–22 g were held in individual stainless steel cages in an air-conditioned room under a 12:12 LD cycle. A commercial pellet diet and water were provided throughout the experiment. P.R. China legislation no. 8910M047 and the guidelines of the Institute for Experimental Animals, Zhejiang University, were adhered to in the use and care of laboratory animals.

Mouse experiments

Prior to treatment, the mice were fasted overnight. The mice were randomly divided into three groups of 10–15 animals in each group; the 1st and 2nd groups orally received 0.5 ml of hemolymph containing OFP-expressed three times a day and the 3rd group (control) of mice received an equivalent amount of the normal worm hemolymph. After 15 days of oral feeding, the blood samples were drawn from the mice and then the intestines were dissected in cold physiological saline solution. The blood smear and the intestine were examined by CLSM.

Fish experiments

The catfish, *Clarias leather*, were divided into three groups of 10 in each group. The 1st and 2nd groups received 0.5 ml of hemolymph containing OFP- or GFP-expressed orally each day and the 3rd group received an equivalent amount of the normal worm hemolymph (control).

The blood of *Clarias leather* was collected after 20 days of oral feeding. Control and experimental *C. leather* were then sacrificed. The intestine was dissected from control and experimental *C. leather* in cold physiological saline solution. The fish blood was surveyed under a UV microscope and the intestine was observed with CLSM.

Confocal laser scanning microscopy

Observation material was imaged on a glass slide under a cover slip. CLSM was performed with a Leica TCS NT/SP microscope (Leica, Heidelberg, Germany) (Nakamura et al., 2007; Breunig et al., 2008). The excitation and emission values were set as 514 and 570 nm, and their collection wavelengths at 580 to 610 nm for OFP.

The excitation and emission values were 457 and 504 nm and the respective collection wavelengths were 510–545 nm for GFP. Several images were taken at different foci and overlays were produced with the Leica TCS-NT/SP software (version 1.6.587).

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