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Some features of intestinal absorption of intact fibrinolytic enzyme III-1 from *Lumbricus rubellus*

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Abstract

In order to investigate whether earthworm fibrinolytic enzyme III-1 (EFE-III-1) isolated from *Lumbricus rubellus* is capable of transporting into blood through intestinal epithelium and keeping its biological function in circulation, we have raised an antibody against EFE-III-1. The immunological results showed that 10–15% of intact EFE-III-1 was absorbed by the intestinal epithelium with the incubation chamber method [Vilhardt and Lundin, Acta Physiol. Scand. 126 (1986) 601–607]. The enzyme could be detected in the intestinal epithelial cells by immunohistochemistry. Furthermore, immunoreactive intact EFE-III-1 was found in serum or plasma after intraperitoneal injection of rats. Approx. 10% of the full-size enzyme could transport through the intestinal epithelium. The maximum remaining activity in blood could be assayed around 60 min after the intraperitoneal injection.

Keywords: Earthworm fibrinolytic enzyme; Protease; Horseradish peroxidase; Intestinal absorption; Intraperitoneal injection; Immunohistochemistry; Immunoblotting; ELISA

1. Introduction

Recently, Nakajima et al. reported that extracts of an earthworm, *Lumbricus rubellus*, contained six different fibrinolytic isoenzymes (EFEs) [1]. EFE-III-1 and III-2, two of the isomers, had strong fibrinolytic activities, broad pH optima (pH 9–11) and resistance to thermal and guanidine-HCl denaturation [2,3]. These features were effective and useful for EFEs to treat some clotting diseases. EFE-III-2 [4] was experimentally inactivated (95%) when incubated with rat plasma at 37°C in 20 min. Mihara et al. [5] found that the enzymes activated the endogenous fibrinolysis system by oral administration. According to some previous reports, a model of pulmonary embolism of rabbit using ^{125}I-labeled fibrinogen showed that EFEs by oral administration had a significant fibrinolytic effect on clots in blood vessels [6]. Thus, it is worth investigating whether intestinal epithelium could absorb the intact and active EFEs. A number of antigenic macromolecules can penetrate the intestinal membrane in their full sizes, such as α-lactalbumin [7], hepatitis B surface antigen [8], bromelain [9] and epoxy-β-carotenes [10]. This paper is concerned with penetration of active and full-sized EFE-III-1, through small intestinal epithelium.

2. Materials and methods

2.1. Materials

We isolated EFEs from the earthworm (*L. rubellus*) and then purified EFE-III-1 from the six homogeneous enzymes, as described previously [3]. The purified EFE-III-1 showed a single band in SDS–PAGE (30 kDa) with specific activity of 48 U (1 U is defined as the specific activity required to convert 1 μM substrate/min/mg of enzyme) [11]. It was lyophilized and stored at 4°C before use. The activity did not change over 6 months. CNBr-activated Sepharose 4B and nitrocellulose membrane were from Pharmacia (Piscataway, NJ, USA). Chromozym TH was obtained from Boehringer (Mannheim, Germany).

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Plasminogen, thrombin, 3,3′-diaminobenzidine (DAB) and fibrinogen came from Sigma (St. Louis, MO, USA). Both complete and incomplete Freund's adjuvants were the products of Life Technologies (Gaithersburg, MD, USA). Goat anti-rabbit IgG-conjugated horseradish peroxidase (HRP) was from Jackson Inc. Rabbit polyclonal antibodies, anti-human lysozyme and anti-human α-1-trypsin were from ZYMED (San Francisco, CA, USA). Serum and plasma from male Wistar rats weighing approx. 200 g was used for assay of the enzyme. As described in the text, we prepared anti-EFE-III-1 serum by immunizing male New Zealand rabbits. The other reagents used were all analytical grade without further purification. Sections were sliced on a Heidelberg-500 microtome (Germany) and visualized under a Nikon FXA microscope. A Bio-Rad 3550 microplate reader was used for ELISA. Absorbance and light scattering were measured on a spectrophotometer of PE-λ12 and a fluorescence spectrophotometer of Hitachi-F4500, respectively. A desktop scanner (Hewlett-Packard Scanjet II-P) scanned the densities of gray shades of protein bands on nitrocellulose membrane. Centricon-30 (Amicon Inc., USA) was employed for centrifugal ultrafiltration.

2.2. Preparation of purified polyclonal antibody of EFE-III-1

We raised antiserum against EFE-III-1 from New Zealand White male rabbits. The antiserum titer after the fourth injection was detected by ELISA [12]. The enzyme (5 mg) was dissolved in a buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and then coupled to CNBr-activated Sepharose 4B overnight at 4°C, according to the manual of Pharmacia. The coupled column was washed five times with alternating 0.1 M acetate buffer containing 0.5 M NaCl (pH 4.0) and 0.1 M Tris–HCl containing 0.5 M NaCl (pH 8.0) at room temperature until residual activity of EFE-III-1 in the flow through could not be detected. Assays were performed using Chromozym TH as described by Zhou et al. [13]. The enzyme-coupled gel was stored in the presence of 0.03% NaN_3 at 4°C. Purification of the antiserum was attempted to increase the titer. Antiserum (1 ml) was added to the enzyme-coupled column (1×15 cm). The column was equilibrated with 0.1 M Tris–HCl buffer (pH 8.0), and then washed with the same buffer five times before elution with 0.1 μM glycine-HCl (pH 2.3), containing 0.15 M NaCl at 25°C. The fractions of the antiserum were concentrated by ultrafiltration with Centrion-30.

2.3. Intestinal preparation

Rats were fasted more than 36 h before being killed. The preparation of duodenum segment and experiment of EFE-III-1 absorption with incubation chamber were as described by Vilhardt and Lundin [14]. EFE-III-1 (final concentration, 10 μM) was added to Krebs solution at the mucosal side. An aliquot (5 μl) was taken from the serosal side for immunoblotting at different time intervals.

2.4. Cryosection and visualization

After incubation of intestinal segment with EFE-III-1 at the mucosal side for 30 min, the duodenum segment was cut and fixed in 2% polyformaldehyde at 4°C overnight. Then the frozen section (40 μm in thickness) was prepared on a microtome as described by Georgopoulou et al. [8]. According to Ausubel et al. [12], the sections were incubated with anti-EFE-III-1 (diluted 1:200), followed by addition of goat anti-rabbit IgG conjugated with HRP (diluted 1:2000). They were visualized by DAB (10 mg/ml) before being examined under microscope. Sections of intestinal segments, incubated with either phosphate-buffered saline (PBS) instead of anti-EFE-III-1 or Krebs solution alone, were used as a control respectively.

2.5. Intraperitoneal injection and immunoblotting procedure

After being fasted for 36 h, rats were injected with 1 ml EFE-III-1 (1 mg/ml in physiological saline) into their abdominal cavities. The same volume of saline was used as a control. The procedure of immunoblotting referred to 'Molecular Cloning' (Lab Manual) [15]. Aliquots of blood were drawn from rats at different time intervals after intraperitoneal injection. They were diluted (1:4) with double distilled water before being run in 15% SDS–PAGE. Anti-EFE-III-1 (1:250) and the secondary antibody (1:5000) were used. Samples were visualized with DAB in PBS containing 0.015% hydrogen peroxide. The gray densities of staining bands were measured by a HP Scanner [16]. In addition, samples from the incubation chamber were immunoblotted according to the same procedure.

2.6. ELISA test

During chamber incubation [14], 10 μl of the medium from the serosal side was taken at different time intervals. ELISA, as described by Ausubel et al. [12], was used to quantify the absorbed EFE-III-1. Rabbit polyclonal antibodies, anti-human lysozyme and anti-human α-1-trypsin were directly added into the mucosal side as absorption controls. Their dilutions (1:200) were the same as that of anti-EFE-III-1.

2.7. Measurements of EFE-III-1 activity

Chromozym TH as a substrate [11] was used to assay EFE-III-1. When rats were injected intraperitoneally, blood samples were taken at different time intervals and incubated with 3.8% sodium citrate. Plasma was obtained by centrifugation (3000 rpm, 10 min, 4°C) and then used for the assay.

3. Results

3.1. Preparation of polyclonal antibodies against EFE-III-1

Either to prepare an antibody or to study transportation of a protein through intestinal epithelium, purified EFE-III-1 was needed. The purified enzyme indicated a single band both on SDS–PAGE stained by Coomassie brilliant blue (Fig. 1c) and on the nitrocellulose membrane stained with Ponceau S (Fig. 1a). The specific activity was approx. 48 U, assayed with Chromozym TH ($\varepsilon_{405nm} = 9.75\ mM^{-1}\ cm^{-1}$).

Originally, the titer of anti-EFE-III-1 serum raised from rabbits was 1:2000, detected by ELISA. In order to increase the titer, we prepared the affinity column with the immobilized EFE-III-1 coupled to Sepharose 4B to purify this antiserum. The residual activity of the immobilized EFE-III-1 was approx. 50% of the native enzyme, with a broader pH optimum (pH 5.0–11.5) and stronger resistance to heat (up to 70°C) [6]. The titer rose to 1:3000 after purification with affinity chromatography, followed by centrifugal ultrafiltration. Both EFEs and EFE-III-1 were used to test the antibody by Western blotting (Fig. 1b). A specific band (30 kDa) was found on the nitrocellulose membrane in immunoreactive color development. However, the other five homogeneous proteins of EFEs were not distinguishably stained under the same conditions. This shows that the antibody was able to recognize and react with EFE-III-1. Furthermore, the purified antibody obviously inactivated EFE-III-1 (Table 1), suggesting that the epitope recognized by the antibody may be in situ at or near the active site of the enzyme.

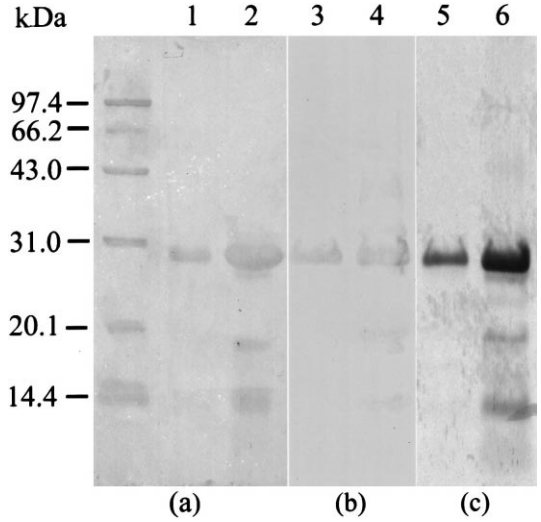

Fig. 1. Western blot with anti-EFE-III-1. 5 μg of purified EFE-III-1 (lanes 1, 3 and 5) and 15 μg of EFEs (lanes 2, 4 and 6) were loaded to each well on 15% SDS–PAGE, which were transferred to the nitrocellulose membrane followed by (a) coloration with Ponceau S and (b) immunoblotting with EFE-III-1 antibody. (c) SDS–PAGE stained with Coomassie brilliant blue.

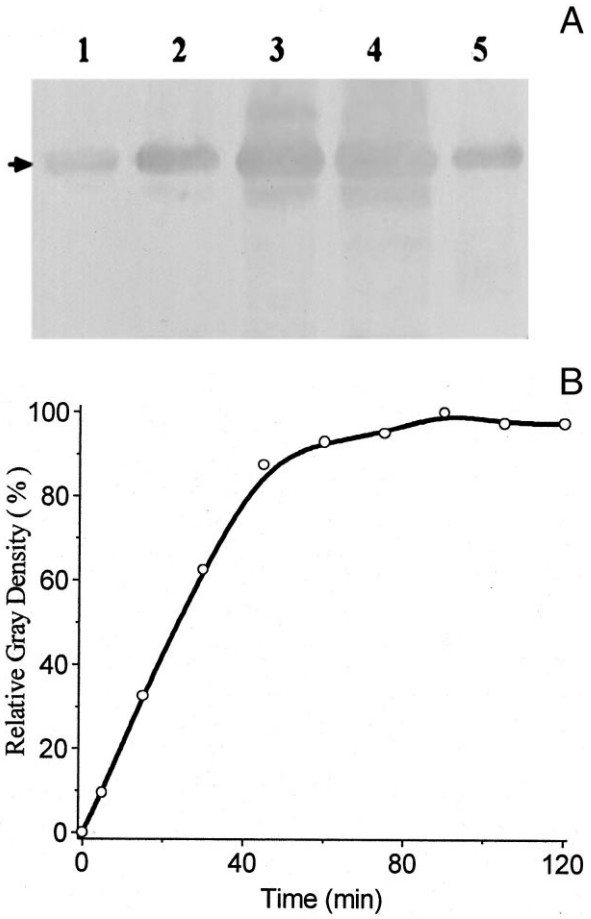

Fig. 2. Mucosal-to-serosal transport of EFE-III-1 during incubation of everted intestinal segments. Aliquots (5 μl) of serosal medium were taken at different incubation time intervals and examined with immunoblotting. (A) Presence of full-sized EFE-III-1 (arrowhead) in serosal medium at incubation times of 5, 30, 60 and 120 min (lanes 1–4). EFE-III-1 as a positive control (lane 5). (B) Relative gray densities of the immunoreactive bands at different times were measured by scanning.

3.2. Intestinal absorption of EFE-III-1

The everted sac model [14,16,17] for studying intestinal transport of large peptides and proteins has been previously described and used validly. On the basis of this method, EFE-III-1 was added into Krebs solution at the mucosal side of a rat small intestinal segment (duodenum). Firstly, we detected the mucosal-to-serosal transportation of the enzyme by immunoblotting. Aliquots of serosal medium were taken at different time intervals during the

Table 1
Effect of the purified anti-EFE-III-1 on activities of EFE-III-1

Antibody (μM)	0	0.05	0.1	0.4
Specific activity (U)	48	36	22	4
Relative activity (%)	100	74	45	7

EFE-III-1 (0.2 μM) was mixed with the purified antibody at different concentrations of 0, 0.05, 0.1 and 0.4 μM in 0.1 M Tris–HCl containing 0.5 M NaCl (pH 8.0). Then the mixture (50 μl) was assayed at 25°C using Chromozym TH as the substrate.

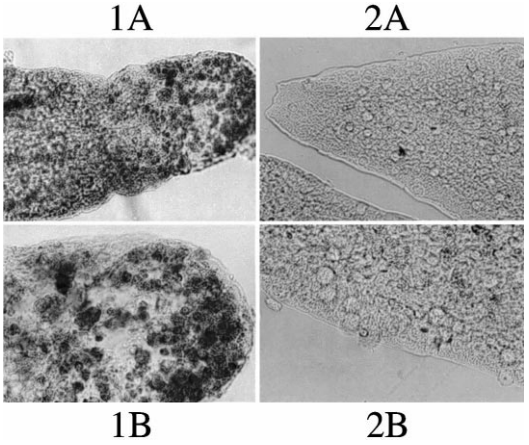

Fig. 3. Immunohistochemistry in intestinal epithelium after incubation of everted sacs with EFE-III-1. After incubating everted intestinal segment with EFE-III-1 (final concentration, 10 μM) at the mucosal side for 30 min, the intestinal sections were immunologically visualized. Specific visualization was present in the intestinal epithelial cells (1A,1B). Control sections were incubated without anti-EFE-III-1 (2A,2B). A: ×100 and B: ×400.

incubation. As shown in Fig. 2A, the immunoreactive bands of EFE-III-1 (30 kDa) were observable during the incubation course, indicating that the intact EFE-III-1 can transport from the mucosal to serosal side. We estimated the quantity of transported EFE-III-1 by scanning the gray shade density of immunoreactive bands on membrane [18]. The densities at different time intervals revealed an increase of EFE-III-1 concentrations in the serosal medium with time (Fig. 2B). Apparently, a transporting maximum appeared around 60 min of incubation. Compared with the standard immunoreactive density curve under the same conditions (data not shown), the amount of the enzyme absorbed and discharged into the medium was approx. 10–15%.

Results of cryosections with immunohistochemistry showed that the significant stained materials had located in the intestinal epithelium after incubation with EFE-III-1 at the mucosal side (Fig. 3, 1A,1B). However, the control sections, which were incubated with the secondary antibody alone, could not be immunochemically stained with DAB (Fig. 3, 2A,2B). A few stained materials, as can be seen, located in some cells on the control sections, might be due to some of the white blood cells which contain HRP.

Furthermore, the control intestinal segments without EFE-III-1 incubation could not be colored with DAB (data not shown) after adding the antiserum and the secondary antibody. We stained the sections with 0.5% cresol purple and 1% toluilene red and compared them with the cryosections by immunohistochemistry. The immunoactive materials were located in the epithelial cells. But no EFE-III-1 remained in intercellular substance. Somehow, the cell organelles involved in containing the stained materials, such as lysosomes or peroxisomes, need further study.

Kinetics in EFE-III-1 intestinal absorption with ELISA (Fig. 4A) showed that the penetration had a relaxation phase because the enzyme could not be detected at the serosal side until 15 min. Consequently, the EFE concentration arrived at the maximum at about 60 min (Fig. 4B). The first order rate of increase in absorption at 415 nm was about 1.11×10^{-3} s^{-1}. On the basis of the standard curve (Fig. 4C), the absorption rate was $k=5.0\times10^{-8}$ M

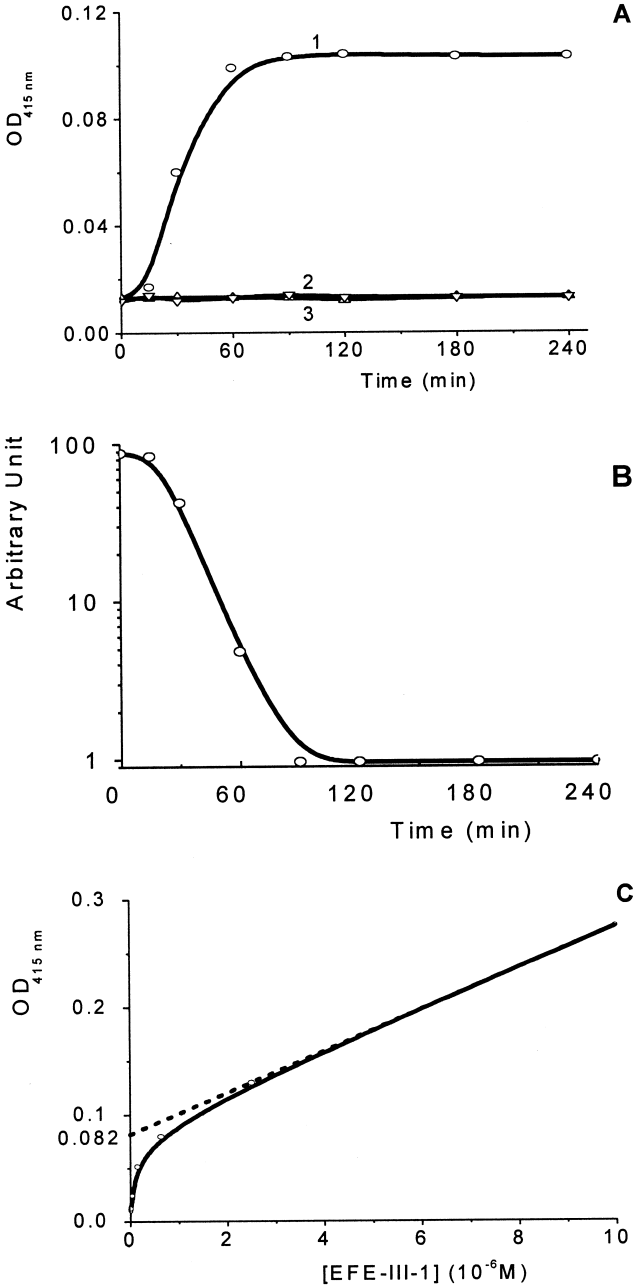

Fig. 4. The time course of EFE-III-1 transportation from the mucosal to the serosal side. (A) Conditions were as for Fig. 3, except the absorbance at 415 nm was measured for ELISA, following that EFE-III-1 (final concentration, 10 μM) was added to the mucosal side (curve 1). Anti-EFE-III-1 and anti-rabbit IgG conjugated with HRP from goat were used as primary and secondary antibodies, consequently. Both anti-lysozyme (curve 2) and anti-α-1-trypsin (curve 3) were used as controls. (B) The same data were plotted in semilogarithmic form. (C) Standard curve of EFE-III-1 in ELISA.

s^{-1}. Thus, we could deduce the maximum transportation of mucosal-to-serosal side at 60 min was as high as 10–15% of EFE-III-1 added at the mucosal side. Both anti-human lysozyme and anti-human α-1-trypsin, as controls, showed no absorption detected by ELISA.

3.3. EFE-III-1 absorbed into blood by intraperitoneal injection

Although EFE-III-1 was detected in the intestinal epithelium, it was necessary to investigate whether the enzyme could be absorbed into blood in vivo. It has been reported that some proteins, for example the injected urokinase, were degraded rapidly by the clearance system and inactivated by some inhibitors in blood [19]. Furthermore, a proportion of proteins would be removed from blood during sample collection and experimental handling [20]. Hence, before intraperitoneal injection, it was necessary to make sure whether the immunological technique was effective to detection of EFE-III-1 in plasma. Firstly, we injected EFE-III-1 into rat muscle, followed by bleeding at different desired time intervals, and detected the enzyme in each aliquot by immunoblotting. An immunoreactive band (30 kDa) appeared on the nitrocellulose membrane, but the control did not show any positive signals (data not shown). This is to say the immunoblotting method was convincing to investigate the presence of EFE-III-1 in blood.

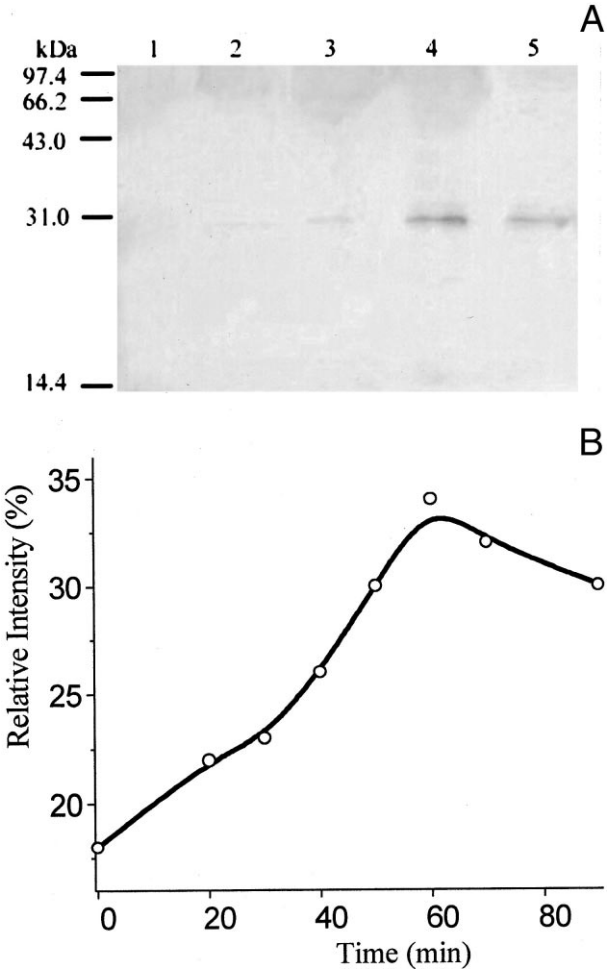

Fig. 5. Immunoblotting of EFE-III-1 in plasma by intraperitoneal injection. (A) After intraperitoneal injection of 1 mg EFE-III-1, rat plasma aliquots were drawn at different time intervals. Lanes 1–5 represent serum samples taken at 0, 20, 40, 60 and 120 min, respectively. (B) Time course of gray densities of immunoblotting bands.

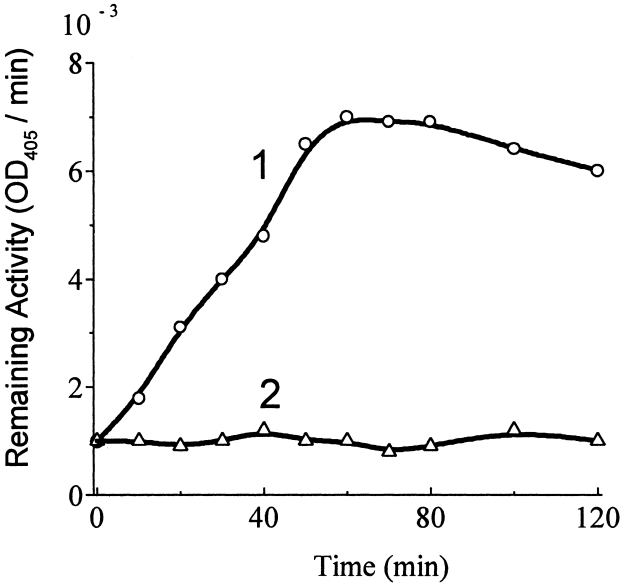

Fig. 6. EFE-III-1 activity in plasma after intraperitoneal injection. After injection of 1 mg EFE-III-1 (curve 1) or physiological saline as a control (curve 2) into rats, plasma aliquots (10 μl) were taken at different time intervals, followed by assaying EFE-III-1. Each value represents the mean of at least three independent measurements.

It is known that intraperitoneal injection has been used to study protein transportation in vivo [21], instead of intestinal absorption by direct administration. We employed rats with this method in our experiments. A single protein band on the nitrocellulose membrane was detected by an immunoblot of the plasma samples (Fig. 5A). And similar results were obtained when serum instead of plasma was taken from rats (data not shown). But nothing was detected by injection of physiological saline as a control. The gray shade density results showed an increase of EFE-III-1 concentrations in plasma with time (Fig. 5B). The maximum density did not appear until 60 min after the injection. The enzyme, absorbed into blood, was estimated to be approx. 10% of the total injection. The immunoblotting experiments suggest that a full-sized EFE-III-1 can be transported from the intraperitoneum into blood.

3.4. Activity of the absorbed enzyme

In order to investigate whether EFE-III-1 could still keep, at least in part, the biological function after being transported from the intestinal peritoneum into blood, we measured the activity by Chromozym TH (Fig. 6). An

increase in the activity could be detected after the injection, with a maximum around 60 min. However, similar to EFE-III-2 [4], EFE-III-1 was inactivated to 5% of the initial activity, indicating that some inhibitors in plasma may interact with this enzyme. The plasma from the rats injected with saline as a control did not show any EFE activities.

4. Discussion

So far, according to some authors, it is accepted beyond a reasonable doubt that macromolecules can be absorbed in intact and biologically active form [7,8]. However, there still exist some barriers in the research of this project because the absorbed proteins are rapidly cleared from blood before they can be detected [9,10]. This problem can arise as a result of one of the following: (i) the active substance is not absorbed in sufficient quantities; (ii) the active substance is present in the blood stream for only a short time or (iii) the active substance forms complexes with blood components [22]. Each of these possibilities comes into question in the case of protease administration. To circumvent this problem, some approaches have been devised and commonly used in these kind of studies, such as incubation chambers in vitro, chromatography, ^{125}I-radiolabeling technique, cytochemical and immunohistochemical methods [8,10,14,16,17,21,23]. It is reasonable for us to employ both intraperitoneal injection and the incubation chamber for our experiments.

In previous studies, some authors used the ^{125}I-radiolabeling technique to investigate the intestinal absorption of a full-sized protein. However, the chemical modification of the protein during labeling and the release of ^{125}I during intestinal digestion might interfere significantly with the result [9]. In this research, the immunological technique is thus employed and the results obtained are reproducible. The quantity of the protein absorbed could be determined by either ELISA [12] or gray shade scanning [16]. The standard curve has suggested that the gray density of the band retained on the nitrocellulose membranes is linearly related to the quantity of the enzyme found in blood.

The research of protein absorption is attracting molecular biologists and pharmacologists to elucidate the mechanism. Walker and Isselbacher have proposed the mechanism of an endocytotic–exocytotic process in the study of the absorption of the intact protein HRP [24]. It is also suggested that transepithelial transport can occur by the paracellular route as well as through the cell [25]. In the intracellular transport, some proteins bind to receptors on the surface of the intestinal epithelium, which then invaginate to form phagosomes engulfing the proteins. Some proteins fuse to form phagolysosomes (such as lysosomers) in which digestion might occur. The significant stained materials locate in the intestinal epithelial cells, and the main passage of transportation of EFE-III-1 is an intracellular process. Being a strong protease, EFE-III-1 is resistant to degradation by some cellular enzymes [7]. Thus, it could be discharged in intact form and across the cell membrane by exocytosis.

Recently, it has been reported that the N-terminal sequence of some proteins might affect protein transportation [26]. Efficient transportation of β-galactosidase, which is not able to pass through the cell membrane, can be achieved when an N-terminal 11-amino-acid protein transduction domain (PTD) is added from the human immunodeficiency virus TAT protein according to Schwarze et al. [21]. So, the macromolecule absorption may not be nonselective as has been suggested before. We are interested in some N-terminal sequences of the transportation proteins, such as trypsin [27], PTD [28], bromelain [29], HRP [30] and EFE-III-1 and -2 [2]. These sequences are rich in hydrophobic amino acid residues, which may contribute to the membrane transportation.

EFE-III-1	I-V-G-G-I-E-A-R-P-Y-E-F-P-W-Q-
EFE-III-2	I-V-G-G-I-E-A-R-P-Y-E-F-P-W-Q-
Trypsin	I-V-G-G-Y-T-C-G-A-N-T-V-P-Y-Q-
Bromelain I	A-E-Y-G-R-V-Y-K-D-N-D-E-K-M-R-
Bromelain II	A-S-K-V-Q-L-V-F-L-F-L-F-L-C-V-
HRP	Q-L-T-P-T-F-Y-D-N-S-C-P-N-V-S-
PTD	M-E-P-V-D-P-R-L-E-P-W-

Definitely, the similarity of the N-terminal 15-amino-acid sequences between EFE-III-1 and -2 is 100%. Here, we suppose that EFE-III-2 is also able to transport into blood through the intestinal wall.

Acknowledgements

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