

## IMMOBILIZED EARTHWORM FIBRINOLYTIC ENZYME III-1 WITH CARBONYLDIIMIDAZOLE ACTIVATED-AGAROSE

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Earthworm fibrinolytic enzyme III-1 (EFE-III-1) was prepared to couple with cross-linking agarose activated by 1,1'-Carbonyl-diimidazole (CDI) in this study. Although the activity of the immobilized protease decreased to approximately 64% of the native enzyme, the activity of EFE-III-1 coupled with the resin activated by CDI was higher than that activated by cyanogen bromide (CNBr). The immobilized protease was experimentally demonstrated to hydrolyze IgG, albumin and creatine kinase, besides fibrin(ogen) and plasmin(ogen), suggesting that EFE had a broad substrate specificity.

**KEYWORDS:** immobilization, earthworm fibrinolytic enzyme, carbonyl-diimidazole, dioxane

\* Corresponding author. EFE (earthworm fibrinolytic enzyme), CDI (1,1'-carbonyl-diimidazole)

### INTRODUCTION

Ever since Axen et al. used cyanogen bromide (CNBr) as a matrix-activating agent in the preparation of an immobilized ligand [1-2], CNBr has been widely used not only in affinity chromatography, but also for immobilization techniques. After that, some other useful agents, such as 1,1'-carbonyl-diimidazole (CDI) [3] and epoxide [4], were introduced to activate matrix. However, in some cases, the use of CNBr leads to ion-exchange effects that were so extensive that non-specifically bound proteins continually obliterated the specific affinity interaction with the target molecules [5]. In some epoxide cases, a charged matrix was avoided, but the gel manifested insufficient capacity due to low coupling yields with ligands.

Recently, earthworm fibrinolytic enzymes (EFEs) were isolated and made into an oral medicine to treat clotting diseases [6-8]. In fact, EFEs contain six isozymes, acting with a serine-protease like function. It was, however, necessary to investigate if EFEs could hydrolyze some important proteins in serum, besides degrading fibrin(ogen) and plasmin(ogen) in anti-coagulation [9]. In this paper, CDI-activated-Sepharose CL-6B was used to immobilize EFE-III-1 that could hydrolyze some important proteins, such as antibody, albumin and creatine kinase.

## MATERIALS AND METHODS

### 1.1. *Materials.*

BSA (fraction V) and Chromozym TH (Tos-Gly-Pro-Arg-4-NA) were from Boehringer (Mannheim, Germany). 1,1'-Carbonyl-diimidazole (CDI), cyanogen bromide (CNBr) and IgG (Fab fragment) came from Sigma company. Sepharose CL-6B was from Pharmacia Company. Dr Li-Xiang Hou in the Institute of Biophysics (CAS) kindly provided rabbit muscle creatine kinase (CK) [10]. We isolated EFEs from the earthworm (*L. rubellus*) and then purified EFE-III-1 from the six homogeneous proteins as described previously [11]. The purified EFE-III-1 showed a single band in SDS-PAGE (~30 kDa) with a specific activity of 48 units (one unit was defined as the specific activity required to convert 1  $\mu$ M substrate/min per mg of enzyme) [12]. Then it was lyophilized and stored at 4°C before use. The activity of the lyophilized EFE did not change when frozen for a period of 6 months. All other reagents used were analytic grade without further purification. Absorbance was measured on a PE -7 spectrophotometer.

### 1.2. *Activation of the resin.*

Before performing the immobilization, gradient dioxane solutions (A, B and C) were prepared at different volume ratios of dioxane/water: 2/8 (A), 5/5 (B) and 8/2 (C), respectively. With a sintered filter funnel, Sepharose CL-6B (1.0g) was sequentially suspended in solutions A, B and C, and finally suspended in absolute dioxane (10 ml) before CDI was added. The resin was incubated with CDI (0.4 g) at 25°C, and slightly shaken for 15 min. The activated matrix was washed with absolute dioxane (5 times in volume), and then was washed in queues with solutions C, B, A and double distilled water (5 times in volume to the resin), respectively. The activated matrix was dried into powder by evacuation on a filter-flask at room temperature before use. The dried powder could be kept at 4°C over 3 months. In addition, CNBr was also used to activate Sepharose CL-6B as described by Wilchek et al. [13], and it was also kept in powder.

### 1.3. *Immobilization of EFE-III-1.*

The CDI(or CNBr)-activated Sepharose CL-6B was swollen with distilled water at a concentration of 0.1 mg/1.8 ml at room temperature for 30 min, and then washed on a sintered glass filter. EFE-III-1 (2.4 mg) dissolved in 0.6 ml buffer (0.1 M NaHCO<sub>3</sub>, 0.5 M NaCl, pH 8.3) was coupled to the matrix overnight at 4°C according to the Pharmacia manual. The product was washed for five cycles at room temperature, alternating between 0.1 M acetate buffer (containing 0.5 M NaCl, pH 4.0) and 0.1 M Tris-HCl buffer (0.5 M

NaCl, pH 8.0), until the residual activity of EFE-III-1 in the flow through could not be detected. The EFE-III-1 coupled resin was stored in the presence of 0.02%  $\text{NaN}_3$  at 4°C before use. The immobilization of the CNBr-activated resin was performed as described by Wilchek et al.[13].

#### 1.4. Assay of EFE-III-1.

EFE and immobilized EFE were assayed with Chromozym TH, a chromogenic substrate, as described by Zhou *et al.* [14].

#### 1.5. Degradation of Creatine kinase, IgG and BSA.

CK (0.1 mg/ml, final concentration) was incubated with immobilized EFE-III-1 (4  $\mu\text{g/ml}$ , as final concentration) in 0.05 M Gly-NaOH buffer (pH 9.2, 25°C), and aliquots were taken for assay and detection on 10% SDS-PAGE at different time intervals. Assay of CK was carried out as described previously by Hou and Vollmer [15]. Under the same conditions, IgG and BSA were employed in hydrolysis by the immobilized EFE.

## RESULTS AND DISCUSSION

### 2.1. Some properties of immobilized EFE-III-1.

Assays of the immobilized protease (Table 1) showed that the activity of the immobilized protease was approx. 29 units, 64% compared with the activity of the native enzyme. However, a relative low activity (approx. 20 units) was obtained when EFE-III-1 was immobilized onto CNBr-activated matrix under the same conditions. In addition, cyanogen bromide is known to be hazardous, therefore special precautions must be taken during each experiment. Furthermore, the matrix activated with CNBr had ion-exchange effects that were so extensive that non-specifically bound proteins continually obliterated the specific affinity interaction with the target fraction [8]. It was suggested that CDI could be better used as an activating-agent to agarose matrix than CNBr in immobilization of a protease, when Lys was not as an essential group of the protein employed, for instance the earthworm fibrinolytic enzyme.

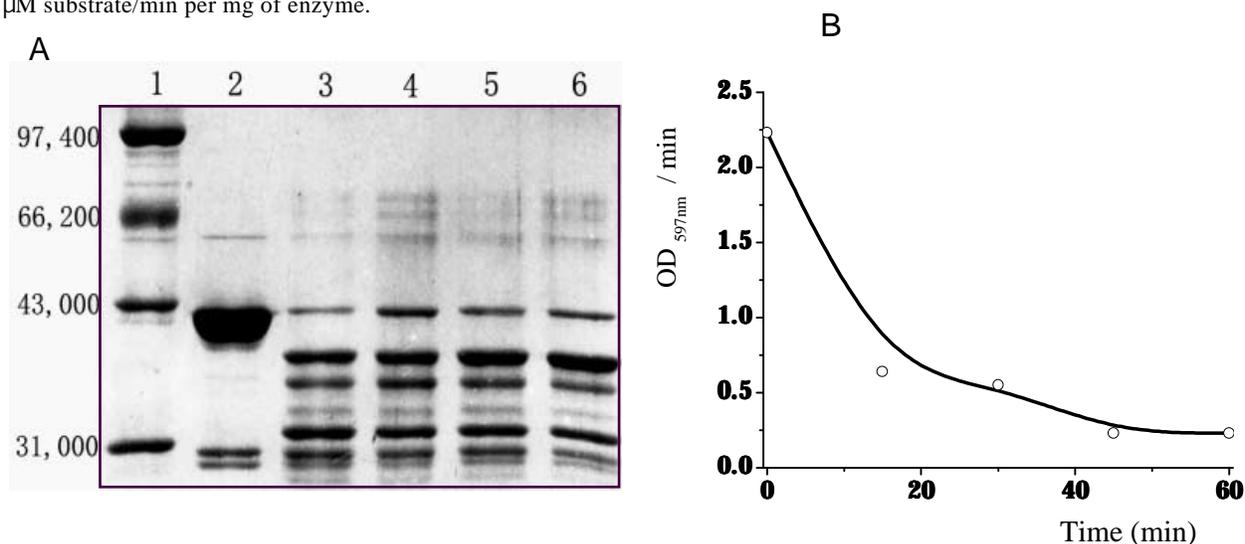
### 2.2. Hydrolysis of CK with the immobilized enzyme.

Here, CK, which was an important enzyme in regulation of cellular energy metabolism, was used as a protein substrate to EFE-III-1. The kinase was hydrolyzed by the immobilized protease into at least 4 fragments (Fig. 1A), accompanied with a distinguishable inactivation (Fig. 1B). Both degradation and inactivation approached to completion in 45 min. It indicated that EFE-III-1, as a serine-protease, had a strong hydrolytic activity.

**Table 1.** EFE-III-1 activity before and after immobilization\*.

Activity of EFE-III-1	CDI-activated	Cyanogen bromide-activated
Activity before immobilized (units)	45	45
Activity after immobilized (units)	29	20
Relative activity (%)	64	44

Assay with Chromozym TH, referred to Zhou et al. [14]. One unit was defined as the specific activity required converting 1  $\mu\text{M}$  substrate/min per mg of enzyme.

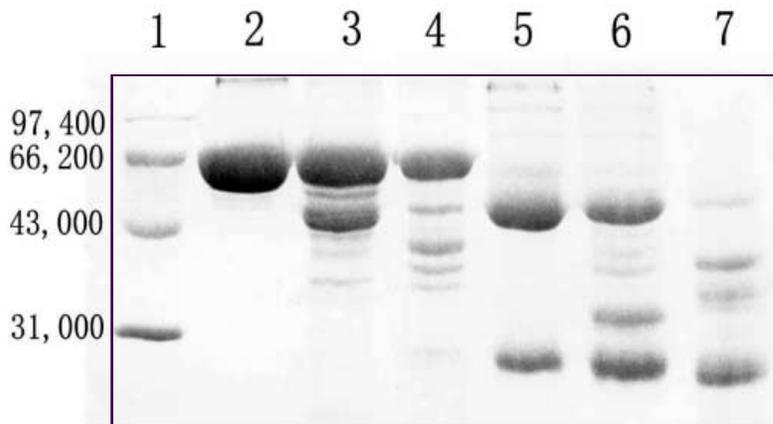


**Figure 1. Hydrolysis of creatine kinase.** CK (0.1 mg/ml as final concentration) was incubated with immobilized EFE-III-1 (4  $\mu\text{g}/\text{ml}$  as final concentration) in 0.05 M Gly-NaOH buffer (pH 9.2, 25°C), and aliquots were taken for assay and detection on 10% SDS-PAGE at different time intervals. Assay of CK was carried out as described previously by Hou and Vollmer [15]. (A) Hydrolysis map of CK. Lane 1 was molecular maker, and Lane 2 through 6 represented CK incubated with immobilized EFE-III-1 for 0, 15, 30, 45 and 60 min, respectively. (B) Inactivation of CK during the hydrolysis.

### 2.3. Hydrolysis of IgG and BSA with the immobilized enzyme.

EFEs have been used as an oral medicine to treat clotting diseases in clinic. There was a concern about whether the oral medicine could degrade some important proteins in serum, such as antibody and albumin. Thus, IgG and BSA (fraction V) were taken as the substrates for EFE-III-1 in hydrolysis experiments (Fig. 2). EFE-III-1 was demonstrated to be capable of hydrolyzing IgG and BSA and it released at least 3 fragments from IgG and 4 fragments from BSA, respectively, suggesting that EFE-III-1 had a broad specificity to its substrates. Recently, a serine residue was found as an essential group at the active site of EFE-III-1 by X-ray crystal-structure (unpublished data, provided by Dr Dong-Cai Liang et al. in this institute) [16], positively

indicating that EFE-III-1 acts with a serine-protease like function. Nevertheless, comparison of the other serine-proteases, the active pocket of EFE-III-1 was observed to have a stronger flexibility that may be a possible explanation for this enzyme having a broader specificity in its substrates.



**Figure 2. Hydrolysis of IgG and BSA.** Hydrolysis conditions were referred to Figure 1, except BSA and IgG (1 mg/ml, as final concentration) were employed. Lane 1 was molecular marker. Lane 2 through 4 represented BSA that was hydrolyzed by immobilized EFE-III-1 for 0.0, 60 and 120 min, respectively. Lane 5 through 7 represented IgG hydrolyzed for 0.0, 60 and 120 min, respectively.

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