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Purification of a Lethal Toxin of *Edwardsiella tarda*

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A toxin lethal to fish was purified from extracellular products (ECP) of *Edwardsiella tarda* by ion exchange chromatography and gel filtration. The purified toxin is a protein having proteolytic activities and lethal toxicity to Japanese eel *Anguilla japonica* with the LD_{50} of 1.6 μg per g of fish body weight. Molecular weight of the protein was estimated to be 37 kDa by SDS-PAGE. SDS-PAGE, Western blot and immunodiffusion analyses revealed that the 37 kDa protein was present in the ECP and ICC (intracellular components) of virulent strains of *E. tarda* but not in those of avirulent strains, indicating that this toxin plays an important role in the pathogenicity of *E. tarda*.

Key words: *Edwardsiella tarda*, toxin, extracellular product, intracellular component, Japanese eel, ECP

Edwardsiellosis caused by *Edwardsiella tarda* has been responsible for significant losses in fish culture industry, particularly in Japanese eel *Anguilla japonica* and Japanese flounder *Paralichthys olivaceus* in Japan (Kusuda and Salati, 1993), Japanese eel in Taiwan (Kou, 1981), and channel catfish *Ictalurus punctatus* in USA (Plumb, 1994). Some candidate virulence factors of the bacterium such as siderophore (Kokubo *et al.*, 1990), hemolysin (Kusuda and Kitadai, 1993; Watson and White, 1979), and resistance to phagocytosis (Iida and Wakabayashi, 1993) have been reported, however, *in vivo* role of these virulence factors in fish is still unknown. Our previous study (Suprapto *et al.*, 1995) demonstrated that *E. tarda* strains produced extracellular products (ECP) lethal to Japanese eel and Japanese flounder and the toxicity of ECP correlated with the pathogenicity of the strains. The lethal toxicity was detected in the ECP at late growth stages on cellophane-overlaid agar media and was also found in the intracellular components (ICC). The present study was conducted to purify the lethal toxic substance of *E. tarda*.

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Materials and Methods

Bacterial strains and preparation of ECP and ICC

Six strains of *E. tarda* isolated from fish or water were used in this study. Four strains, NUF251, E22, SU226, and FPC498 belonging to serotype A, were pathogenic to Japanese eel and their ECPs and ICCs were lethal to the fish, while the other two strains, SU138 (serotype B) and SU100 (serotype C), were avirulent and their ECPs and ICCs were not lethal (Suprapto *et al.*, 1995). These strains were stored in tryptic soy broth (BBL) with 10% glycerol at −80°C and subcultured on tryptic soy agar (TSA, Nissui) at 25°C for 48 h prior to the onset of experiments. The ECP and ICC were prepared from the culture on cellophane-overlaid TSA at 25°C for 96 h according to the method described previously (Suprapto *et al.*, 1995).

Ion exchange and gel filtration chromatography

The ECP of strain NUF251 was used to purify the lethal toxin. The ECP dialyzed overnight against 10 mM phosphate buffer (PB, pH 7.0) was applied to Toyopearl 650 M gel (Tosoh) column equilibrated with PB and proteins were eluted with a linear gradient of 10 to 100 mM PB at a flow rate of 1 ml/min. Protein fractions detected at 280 nm were

collected, pooled, and concentrated approximately 5-fold by polyethylene glycol 20,000. The concentrated fraction of each peak was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and injected to Japanese eel to check the toxicity. The toxic fraction was applied to Sephadex G150 (Pharmacia) column equilibrated with 25 mM PB and eluted at a flow rate of 0.5 ml/min. The fractions were pooled, concentrated, analyzed by SDS-PAGE and tested for the toxicity. Protein contents were measured according to the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Toxicity test

Healthy Japanese eels weighing about 80 g in body weight were used for the examination of lethal toxicity of fractionated proteins. Each protein peak from ion exchange chromatography or from gel filtration was injected intramuscularly at a dose of 600 μg or 500 μg per fish. To determine LD_{50} of purified toxin (peak III in gel filtration), fish were injected at doses ranging from 50 to 200 μg per fish. After injection, fish were kept in 50*l* plastic tanks at 19–23°C and observed for 2 weeks. Bacterial isolation from the kidney of dead fish was attempted to confirm that the death was not due to bacterial infection. The purified toxin was also intracutaneously injected in 5 body sites of a rabbit at a dose of 300 μg/site and the skin reaction was observed for 3 days.

Preparation of antiserum to toxin

A rabbit was injected subcutaneously with 100 μg of the purified toxin emulsified with an equal volume of Freund's complete adjuvant (Difco), followed by four booster injections without adjuvant in the ear vein. The blood was drawn 35 days after the first inoculation and the serum collected was inactivated at 56°C for 30 min. Detection of antibodies against purified toxin and antigenic comparison of ECP and ICC of 6 *E. tarda* strains were made by Ouchterlony immunodiffusion using 1% agarose gel.

Electrophoresis and immunoblot

SDS-PAGE and immunoblot analyses were carried out according to the method described by Laemmli (1970) and Towbin *et al.* (1979), respectively. The concentrated fractions from ion exchange and gel filtration chromatographies were mixed with 0.17 M Tris-HCl buffer (pH 6.8) containing SDS (5.3%) and 2-mercaptoethanol (13.2%). Electrophoresis using 10% gel and Tris (0.025 M)-glycine (0.192 M) buffer containing 0.1 M SDS was performed at a constant volt of 150 for 1 h. The protein bands were stained with Coomassie brilliant blue. After SDS-PAGE, the protein in gel was transferred electrophoretically to a nitrocellulose membrane filter. The membrane was blocked with TBS buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl) containing 2% skim milk. After washing, the membrane was reacted with the anti-toxin rabbit serum diluted 1 : 500 with TBS buffer for 1 h, washed with the buffer, and then reacted with alkaline phosphatase-conjugated anti-rabbit Ig swine Ig (Dako). Finally, the protein bands were visualized by 5-bromo-4-chloro-3-indoryl phosphate (X phosphate) and 4-nitroblue tetrazolium chloride (NBT).

In vitro biological activities of toxin

Proteolytic activities of the purified toxin were assayed according to the method described by Hastings and Ellis (1985). Radial diffusion method using 1% agarose gel was used for the determination of caseinase (3% soy bean casein as substrate) and gelatinase (3% gelatin) activities. After incubation at 25°C for 24 h, plates were flooded with 15% mercuric chloride in 20% HCl. One unit of the activity was defined as that which produced a clear zone equal in area to that produced by 1 μg of trypsin.

Hemolytic activity was measured in a 96-well microplate. Serially 2-fold diluted toxin samples were mixed with 1% red blood cell suspension of Japanese eel and horse. The plate was incubated at 25°C for 3 h.

Cytotoxic effects were examined using three fish cell lines (FHM, EPC, RTG-2). Fifty microliter of the toxin sample was added to a monolayer culture of each cell, which were prepared in 96-well plates with Eagles's MEM (Nissui) supplemented with 10% fetal bovine serum, and the plates were incubated at 20°C for 48 h.

Results and Discussion

Figure 1 shows the elution profile of the ECP of *E. tarda* (NUF251) in ion exchange chromatography. Among five protein peaks, three peaks 3, 4, and 5 were lethal to Japanese eel when injected at a dose of 600 μg per fish (Table 1). Fish died within 3 days post-injection showing similar pathological signs,

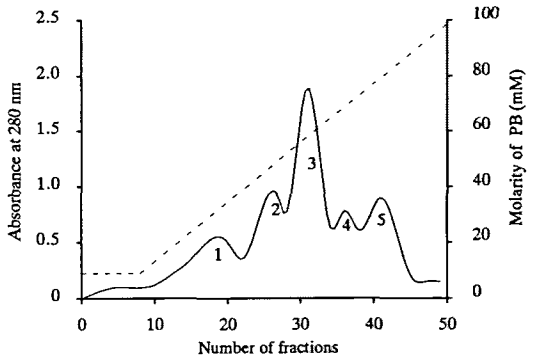

Fig. 1. Elution profile of ECP proteins of *Edwardsiella tarda* (NUF251) in ion exchange chromatography.

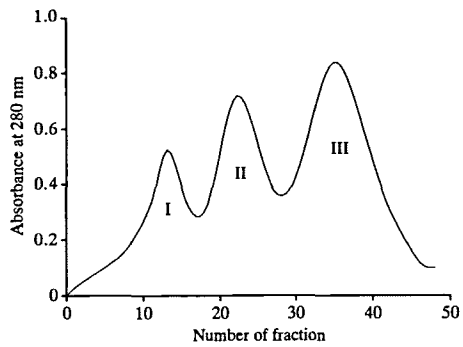

Fig. 2. Elution profile of ECP proteins of *Edwardsiella tarda* (NUF251) in gel filtration (Sephadex G150).

such as hemorrhage on the skin, abscesses in the muscle at the injection site, and discoloration of the kidney, to those observed in the eels injected with ECP (Suprapto *et al.*, 1995). These toxic fractions were then further fractionated by gel filtration (Fig. 2). One protein peak, peak III, consisting of a single band was lethal to eel when injected at a dose of 500 μg per fish (Table 1) and the molecular weight of the protein was estimated to be 37 kDa by SDS-PAGE (Fig. 3A). The LD_{50} of this purified toxin to Japanese eel was calculated as 125 μg/fish or 1.6 μg/g of fish body weight (Table 1).

The Ouchterlony double diffusion with the antitoxin rabbit serum resulted in the formation of a continuous precipitin line between the purified toxin and ECP of NUF251 strain. The same precipitin line was formed with ECPs of 4 virulent strains of *E. tarda* (NUF251, E22, SU226, FPC498) but not with those of 2 avirulent strains (SU100, SU138) (Fig. 4). In SDS-PAGE and Western blot with the antiserum, the 37 kDa protein was detected in the ECPs and ICCs of all the virulent strains, while that protein was not detected in avirulent strains (Figs. 3 and 5).

As demonstrated in a previous study (Suprapto *et al.*, 1995), the production of toxic ECP and ICC correlated with the pathogenicity of the strains in eels. The present results, therefore, indicate that the 37 kDa protein is a lethal substance present in the ECP and ICC of virulent *E. tarda* strains. This means that this protein is one of the virulence factors of *E. tarda*.

The purified toxin exhibited caseinase and gelatin-

Table 1. Toxicity of different protein constituents of *Edwardsiella tarda* (NUF251) ECP to Japanese eel

Sample injected		Dose injected (μg/fish)	Mortality (dead/tested)	Mean time to death (days)
ECP		600	3/3	7
Constituent*				
Ion exchange-peak	1	600	0/3	—
	2	600	0/3	—
	3	600	3/3	3
	4	600	3/3	3
	5	600	3/3	3
Gel filtration-peak	I	500	0/3	—
	II	500	0/3	—
	III	500	3/3	3
	III	200	5/5	3
	III	150	3/5	7
	III	100	2/5	10
	III	50	0/5	—

* Each constituent was obtained by ion exchange chromatography (Fig. 1) and gel filtration (Fig. 2).

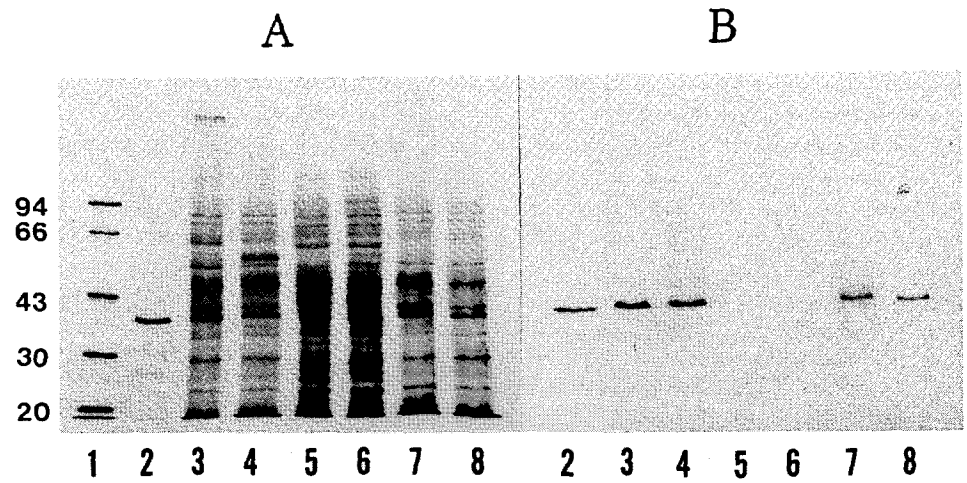

Fig. 3. SDS-PAGE and Western blot analyses of ECP of 6 *Edwardsiella tarda* strains. A: SDS-PAGE, B: Western blot, 1: Marker, 2: Purified toxin (peak III in gel filtration), 3: NUF251, 4: E22, 5: SU100, 6: SU138, 7: SU226, 8: FPC498.

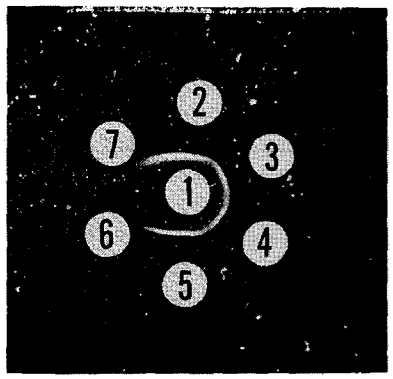

Fig. 4. Immunodiffusion analysis of ECP of 6 *Edwardsiella tarda* strains. 1: Rabbit antiserum against purified toxin (peak III in gel filtration), 2: NUF251, 3: E22, 4: SU226, 5: FPC498, 6: SU 138, 7: SU100.

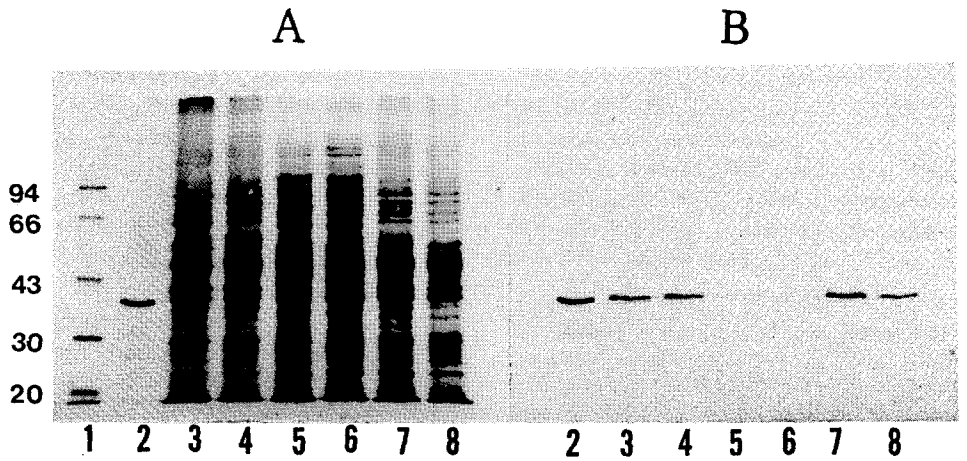

Fig. 5. SDS-PAGE and Western blot analyses of ICC of 6 *Edwardsiella tarda* strains. A: SDS-PAGE, B: Western blot, 1: Marker, 2: Purified toxin (peak III in gel filtration), 3: NUF251, 4: E22, 5: SU100, 6: SU138, 7: SU226, 8: FPC498.

ase activities. Some paper reported the production of hemolysin by *E. tarda* (Kusuda and Kitadai, 1993; Watson and White, 1979), but no hemolytic activity against eel or horse red blood cells was observed in the present lethal toxin, though low hemolytic activity was found in the ECP and ICC. The cytotoxic effect of the toxin for three cultured fish cells (EPC, FHM, RTG-2) was also not observed. Ullah and Arai (1983) reported two kinds of proteinaceous dermatonecrotic substances for rabbit in ECP of *E. tarda* strains isolated from diseased fish, however, such an activity for rabbit was not detected in the present 37 kDa protein. As intracellular parasitic nature is seemingly the most important virulence factor of *E. tarda* (Mamnur Rashid *et al.*, unpublished), it is interesting to know the role of the present lethal toxin in the resistance against phagocytosis of host fish.

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