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Engineering Ecotin for Identifying Proteins with a Trypsin Fold

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Abstract Ecotin is a bidentate, fold-specific inhibitor of mammalian serine-proteases produced by *Escherichia coli*. This molecule may be engineered to increase and/or change its affinity and specificity providing significant biotechnological potential. Since ecotin binds tightly to serine proteases of the trypsin fold, it may help to identify the role of these enzymes in different biological processes. In this work, we tested ecotin variants as an affinity purification reagent for identifying enzymes in samples of tumor progression and mammary gland involution. Initially, we used a commercial source of urokinase-type plasminogen activator (u-PA) that remained fully active after elution from an affinity column of the ecotin variant (M84R, M85R). We then successfully identified u-PA from more complex mixtures including lysates from a prostate cancer cell line and involuting mouse mammary glands. Interestingly, a membrane-type serine protease 1 was isolated from the Triton X-100-solubilized PC-3 cell lysates, and surprisingly, haptoglobin, a serine-protease homolog protein, was also identified in mammary gland lysates and in blood. Haptoglobin does not prevent ecotin inhibition of u-PA, but it may act as a carrier within blood when ecotin is used *in vivo*. Finally, this affinity purification matrix was also able to identify a thrombin-like enzyme from snake venom using an ecotin variant directed against thrombin. Overall, the ecotin variants acted as robust tools for the isolation and characterization of proteins with a trypsin fold. Thus, they may assist in the understanding of the role of these serine proteases and homologous proteins in different biological processes.

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Introduction

Serine proteases possessing a trypsin fold are involved in many biological processes, including blood coagulation [1], metastasis of cancer cells [2], fibrinolysis [3], mammary gland involution [4], and the envenomation process [5]. These enzymes may be inhibited by ecotin, a periplasmic *Escherichia coli*-derived protein and a "fold-specific" inhibitor that has an unusually broad specificity to proteases such as trypsin, chymotrypsin, elastase, factor Xa, kallikrein, and factor XIIa [6, 7]. This inhibitor uses two distinct binding sites to recognize its target and has been engineered to potently and specifically inhibit proteases such as urokinase-type plasminogen activator (u-PA) [6–8]. Literature described that ecotin is able to purify recombinant trypsinogen expressed in *E. coli* [9] although it does not recognize more specific proteases such as u-PA.

Tissue culture cell lines serve as valuable models for cancer and cancer progression. Human PC-3 prostate carcinoma cells have been used as a model of prostate cancer [10]. This cell line expresses serine proteases such as u-PA [11] and the membrane-type serine protease 1 (MT-SP1) [12]. The isolation of proteases such as u-PA and MT-SP1 in different culture cell lines can aid in their characterization. Furthermore, new chromatographic techniques may serve as tools for discovering novel serine proteases derived from cell lines. Of greater complexity would be the isolation of proteases directly from tissue lysates, membranes, or rich mixtures such as involuting mammary glands [13] and snake venom [14], which require fast and reliable techniques.

Proteomics can identify new proteins and help determine their roles in normal physiologic or pathologic processes [15]. The use of ecotin variants that interact with a vast array of serine proteases may aid in isolating and characterizing proteases and homologous proteins from a variety of biological samples. In the present work, we describe the use of variant ecotins for identifying proteins from several biological sources, allowing simple and rapid identification of trypsin-fold proteins.

Materials and Methods

Materials

Snake venom from *Lachesis muta,* cyanogen bromide-activated agarose, and haptoglobin (Hp) were purchased from Sigma (St. Louis, MO). PC-3 (CRL-1435) cells were obtained from American Type Culture Collection (Rockville, MD) and grown according to the supplier's instructions. Human urokinase-type plasminogen activator, SPECTROZYME®UK substrate, and anti-u-PA polyclonal antibody were obtained from American Diagnostica (Greenwich, CT). All other reagents were purchased from Sigma or Fisher Scientific unless otherwise noted.

Preparation of Ecotin Affinity Column

Ecotin variants used in this study were ecotin-RR (M84R and M85R) constructed against u-PA and ecotin-TSRR/R (V81T, T83S, M84R, M85R, and D70R) against thrombin and u-PA. They were purified from *E. coli* as described [5].

Affinity columns were prepared by incubating the engineered ecotin (45 mg) with cyanogen bromide-activated agarose (15 mL) overnight in 100 mM Na bicarbonate (pH 8) at 4 °C. The ecotin column was washed extensively with phosphate-buffered saline (PBS) pH 7.5, and any unbound ecotin was washed out using 50 mM glycine, 100 mM NaCl, pH 3.0. The final column was equilibrated with PBS and stored at 4 °C.

Sample Preparation

Standard Serine Protease

Human urokinase-type plasminogen activator (3,000 IU) was diluted in 3 ml of PBS and applied directly to the ecotin-RR column.

Conditioned Media of Prostate Cancer Cells

The PC-3 (CRL-1435) cell line was grown at 37 °C in a serum-free medium in a 100-mm dish, and the supernatant was collected when cells had reached 90% confluency. This material (40 mL) was dialyzed against PBS, concentrated fourfold, and stored at 4 °C until being applied directly to the ecotin-RR column.

PC-3 Cell Lysates

The PC-3 cell line was grown to 90% confluency as listed above. The cells on the plate were washed twice with PBS. After washing, the cells were lysed with 1% Triton X-100, 5 mM EDTA in PBS. The cell lysate was collected and centrifuged, and the supernatant (10 mL) was collected and applied to an ecotin-RR column.

Mammary Gland Cells

Female CF1 mice were crossed with CD1 males (Charles River Biologicals, Wilmington, MA) and allowed to undergo a normal pregnancy. The pups were weaned following 7–10 days of lactation (day 0 of involution), and the dams were killed on day 5 of involution. Freshly isolated mammary tissue was snap frozen in liquid nitrogen and then homogenized in RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS), and the supernatant was collected as described [13]. The supernatant (10 mL) was dialyzed against PBS and stored at −10 °C until application on the ecotin-RR column.

Snake Venom

L. muta venom (50 mg) was partially purified using a Mono-Q column (H10/10) on a Pharmacia FPLC^{R} system. Proteins were eluted with a linear gradient of 0–1 M NaCl in 20 mM Tris pH 7.5 at a flow rate of 2 ml/min. Fractions with the major proteolytic activity were pooled (10 mL), exhaustively dialyzed against PBS and applied to the ecotin-TSRR/R column.

Affinity Chromatography with Ecotin Column

The column (3 ml) with the ecotin variant bound to the agarose was initially equilibrated using 30 mL of PBS. Then, it was incubated with the protein samples for 1 h at room

temperature in a closed flow system using a Pharmacia pump (0.5 mL/min) followed by washing with PBS (30 mL). Retained fractions were eluted with buffer containing 50 mM glycine and 100 mM NaCl pH 3.0 at 1 mL/min. Fractions were neutralized with 1 M Tris (10 μl/ml). Protein elution was monitored at 280 nm using a UVIKON 860 spectrophotometer. Protein-containing fractions were pooled and concentrated tenfold using a Centricon 10 concentrator (Amicon). The eluted material was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresed (SDS-PAGE) and sequenced and/or analyzed by Western blotting.

PAGE and Western-Blot Analysis

Protein samples were electrophoresed on 10% SDS-PAGE according to Laemmli [16]. For immunoblot analysis, bands were transferred onto a nitrocellulose membrane and treated with Tris-buffered saline with 0.1% (*v*/*v*) Triton X-100 (TBST) containing 5% (*w*/*v*) nonfat dry milk. The membrane was probed with a 1:5,000 dilution of the specific antibody in the same buffer for 1 h and washed three times with TBST. A 1:5,000 dilution of goat anti-rabbit horseradish peroxidase conjugated serum (Pierce) was applied in TBST-1% milk and incubated for 1 h with the membrane. The membrane was washed and antibody-bound protein bands were detected by enhanced chemiluminescence [12].

Substrate Gels (Zymography)

Samples were run on SDS-PAGE containing gelatin 3 mg/mL (dry weight) as a substrate. Gels were washed with 2.5% Triton X-100 for 30 min then incubated with 20 mM Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$ and 0.02% NaN_3, for 12 h at 37 °C. Cleavage of the gelatin by proteases results in clear bands upon staining with Coomassie Blue R250 [12]. Plasminogen gels were prepared by adding plasminogen (50 μg/mL) to the gelatin. For preparation of casein-plasminogen gels, gelatin was replaced with 1 mg/mL casein and 10 μg/ml plasminogen.

N-Terminal Sequencing

Proteins were resolved on SDS-PAGE, electrotransferred to a PVDF membrane, and sequenced on a Perkin-Elmer Procise 491 by the Biomolecular Resource Center of the University of California, San Francisco.

Amidolytic Activity

For urokinase assays, hydrolysis of the chromogenic substrate SPECTROZYME ® UK (Cbo-L-(γ)Glu(α-t-BuO)-Gly-Arg-pNA.2AcOH) was measured using a Thermomax Microplate ELISA Reader (Molecular Devices, Menlo Park, CA). Ecotin-RR (2 nM) was incubated with haptoglobin (2 μM) in 50 mM Tris-HCl containing 50 mM NaCl and 10 mM $CaCl_2$ (pH 8.0). Following a 30-min equilibration at room temperature, two aliquots were taken for analysis. One was subjected to electrophoresis on a nondenaturing gel to observe the ecotin–haptoglobin complex formation as described [17]. U-PA (1 IU) was added to the other aliquot, and the reaction was initiated after 2 min at 37 °C by addition of SPECTROZYME®UK (0.2 mM, final concentration). Absorbance was followed at 450 nm.

Fibrinogen Clotting

Fibrinogen clotting was measured in the Thermomax Microplate ELISA Reader. Ecotin (1–50 μM) was incubated with *L. muta* thrombin-like enzyme (LM-TL; 2 nM) in 10 mM Tris-HCl, 10 mM Hepes, 100 mM NaCl, 0.1% PEG 8000 (pH 7.5). Following a 5-min equilibration at room temperature, the reaction was initiated by addition of fibrinogen 4 mg/mL (dry weight) and monitored at 405 nm as described.

Results and Discussion

Ecotin is a macromolecular inhibitor that interacts with target proteases using two distinct binding sites. These binding sites can be fine-tuned by site-directed mutagenesis to potently and specifically inhibit a specific serine protease [6–8]. In this work, we have evaluated the ecotin variants as affinity chromatography reagents as they inhibit proteases in the low nanomolar to subnanomolar range, suggesting the formation of stable complexes. The inhibitory effects of ecotin also have been observed *in vivo*, suggesting that complex formation can be achieved within rich biological mixtures [12]. The chromatographic efficiency of ecotin variants as affinity reagents was analyzed for different biological samples ranging from partially purified snake venom to tissue lysates.

Initially, we used a commercial source of human urokinase-type plasminogen activator (3,000 IU) suspended in PBS (3 mL) to examine the ability of the ecotin variants to bind proteases when attached to a matrix. U-PA was applied to an ecotin-RR (M84R, M85R) affinity column as described in "Materials and Methods." The enzyme appeared as a diffuse band on SDS-PAGE prior to application on the column, but it was recovered as a distinct band at 52 kDa, after elution at pH 3.0 (Fig. 1a). This band was recognized by u-PA antibodies (Fig.1b) and was also fully active on plasminogen gels after elution (Fig. 1c). About 95% of the original activity was recovered, suggesting that the diffuse material represents contaminants (Fig. 1a). These results show that binding of u-PA to the ecotin-RR column is reversible and suggests that the column will be useful for purification of other serine proteases with a trypsin fold.

U-PA is a protease expressed by the PC-3 cell line, as described elsewhere [11]. The development of techniques to isolate this and other proteases derived from tissue culture cell lines is of interest for further characterizing the role of these trypsin-fold proteases in cancer progression. Thus, we used the ecotin-RR affinity column to test whether u-PA may be identified from the conditioned media of the PC-3 cell line. The supernatant from PC-3

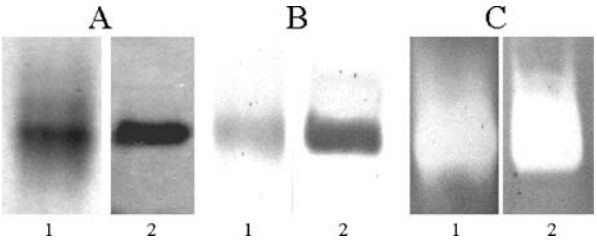

Fig. 1 Analysis of commercial u-PA using ecotin-RR affinity column. SDS-PAGE (**A**), Western blot analysis (**B**), and plasminogen-gelatin gels (**C**) of commercial u-PA before application on the ecotin-RR column (*lane 1*) and after elution at pH 3.0 (*lane 2*). Each lane contains 5 μg of enzyme

cells grown to confluence was concentrated fourfold and loaded onto an ecotin-RR column as described in "Materials and Methods" (Fig. 2a). The eluted material appeared as one band of approximately 52 kDa on SDS-PAGE and was also observed on a gelatin-plasminogen gel and by Western blotting using u-PA antibodies (Fig. 2a, inset). The N-terminal sequence of this band also confirmed it as u-PA (Table 1). This result shows that the affinity column is effective at separating this enzyme from a mixture of proteins secreted into the culture medium by these cancer cells.

Different methods have been used to better understand ecotin binding to serine-proteases including site-directed mutagenesis, enzyme kinetics, and X-ray crystallography [18–21]. These techniques have been used to study ecotin structure–activity relationships, as well as the binding mechanism of ecotin to its target enzyme. In these experiments, amino acid substitutions and deletions in the ecotin primary secondary binding sites generated variants with different levels of potency and specificity. Wang and collaborators reported that ecotin M84R/M85R (referred to here as ecotin-RR) exhibited greater affinity to uPA, promoting a more efficient binding mode [22]. Since ecotin specificity can be tuned, it is possible to isolate trypsin-fold proteases with different specificities, providing a useful tool for dissecting the role of proteases in biological systems [6, 8, 23, 24].

The PC-3 cell line also expresses the trypsin-fold protease, MT-SP1 [12]. Unlike u-PA, MT-SP1 is not secreted and remains localized to the extracellular surface. Since ecotin-RR is a subnanomolar inhibitor of MT-SP1, we used the ecotin affinity matrix to isolate MT-SP1 from solubilized cell lysates. Indeed, direct application of PC-3 Triton X-100 extracts (Fig. 2b, lane 1) allowed the capture of three high molecular weight bands (Fig. 2b, lane 2). Immunoblotting with anti-MT-SP1 antibodies confirmed the identity of the 87 kDa band as the full-length MT-SP1 protein (Fig. 2b, lane 3). Thus, ecotin-RR affinity columns allowed the isolation of uPA from the conditioned media and MT-SP1 from the solubilized cell

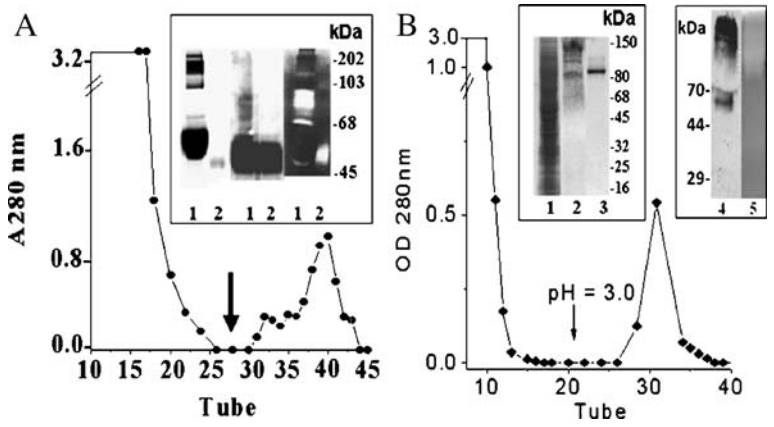

Fig. 2 Analysis of prostate cancer cells (conditioned medium (**A**) and membrane (**B**)) using the ecotin-RR affinity column. **A** Elution pattern of conditioned medium from prostate cancer cells. The graph shows the elution profile (1 mL/fraction) following application of 10 mL of concentrated conditioned medium of untreated cancer cells to a column equilibrated with PBS pH 7.5. *Arrow* indicates the beginning of the elution step using 50 mM glycine in 100 mM NaCl, pH 3.0. *Inset* SDS-PAGE (*left*), Western blot using u-PA polyclonal antibodies (*center*), and plasminogen-gelatin gels (*right*) of untreated medium from cancer cell cultures (*lane 1*) and material eluted at pH 3.0 from the ecotin-RR column (*lane 2*). Each lane contains 8 μg of sample protein. **B** Analysis of eluted material from Tween-extracted cell membrane from Prostate cancer cells (PC_3) on ecotin-RR column. *Arrow* indicates when elution buffer was loaded. *Inset* SDS-PAGE (10%) of extracted cell membrane material (*lane 1*), affinity eluate (*lane 2*), imunoreactivity probed with MTSP1 antibodies (*lane 3*), with trypsin polyclonal antibodies (*lane 4*), and proteolytic activity on gelatin gel (*lane 5*)

Table 1 N-Terminal sequence of proteins from retained material of supernatant of cancer cells, mammary gland cell lysate, and snake venom. Protein identification was determinate using the Swiss Protein Data Base.

Source	Sequence	Identity
Prostate cancer cells	IIGGEFTTIENQPWFFAAIY	u-PA
Mammary gland lysate	IELGNDDAKGEFDWQAKMIS	Haptoglobin α-chain
	VIGGSMAMDFSDPSXPKPPE	Haptoglobin β-chain
Snake venom	VVGGDEXNINEHRXLVLVYX	LM-TL

lysates among other unknown proteins. These unknown proteins were recognized by trypsin polyclonal antibodies and presented proteolytic activity on gelatin gel (Fig. 2b, lanes 4 and 5). In order to identify some of these proteins, a different ecotin mutant column was tested, (M84R and M85P). This affinity column allowed us to identify a 67-kDa band that apparently is the same protein observed in the previous experiment analyzed with trypsin antibodies (Fig. 2b, lane 4). According to our sequencing results, this band refers to trypsin (sequence=IVGGYTCAANSI/VPYQ) bound to a serpin (sequence=TILRIIVRVNRPFLI; not shown). This complex was able to interact with the affinity column because ecotin is able to bind through two binding sites (primary and secondary sites). Therefore, despite trypsin's catalytic site being bound to a serpin, ecotin displaces the serpin or is still able to interact with this enzyme through the secondary binding site. This result is also in agreement with Takeuchi et al (1998) that reported a Trypsinogen IV cDNA sequence in PC3 cells by using molecular biology techniques. Our data infer that this zymogen is probably expressed and activated in the cell in metastasis [12].

Urokinase plasminogen activator has also been implicated in processes such as cell migration and tissue remodeling, which require extracellular proteolytic activity [25]. Since affinity chromatography using ecotin-RR was capable of capturing u-PA in other conditions, we tested whether u-PA could be isolated from lysates of involuting mouse mammary glands. Whole-cell lysate (10 mL) was loaded onto an ecotin-RR column and incubated for 1 h as described in "Materials and Methods" (Fig. 3a). SDS-PAGE showed proteins of about 48 and 80 kDa in the material eluted at pH 3.0 that also exhibited proteolytic activity on substrate gels containing casein and plasminogen (Fig. 3a, inset). Immunoblot analysis with u-PA antibodies recognized the diagnostic 48-kDa band indicating u-PA bound to the column (Fig. 3a inset).

Interestingly, we identified the 80-kDa proteolytic activity as plasma kallikrein, and the N-terminal amino acid sequencing also revealed the presence of Hp in the same band (Table 1). Hp is a tetramer composed of two light chains (α) and two heavy chains (β) linked by disulfide bridges. The heavy β-chain is homologous to members of the mammalian serine protease family [26]. However, the catalytic triad (Ser195, His57, and Asp102) required for enzymatic activity is missing in haptoglobin, resulting in a nonenzymatic protein with a trypsin fold [26–29]. In fact, Hp is an α_2-acidic glycoprotein with hemoglobin-binding capacity, present in most body fluids of humans and other mammals [30]. Biosynthesis of haptoglobin normally occurs in the liver, but it has also been reported to be synthesized in adipose tissue and in the lung with antioxidant and antimicrobial profiles [31, 32]. Although we cannot discard the possibility of contamination from blood during collection of mammary gland cells, the large amounts of haptoglobin that were recovered by the affinity column suggest its expression by the mammary adipocytes.

Haptoglobin's trypsin fold conserves Asp193, homologous to Asp194 of serine proteases that is involved in the conformational change that takes place following proteolytic activation of

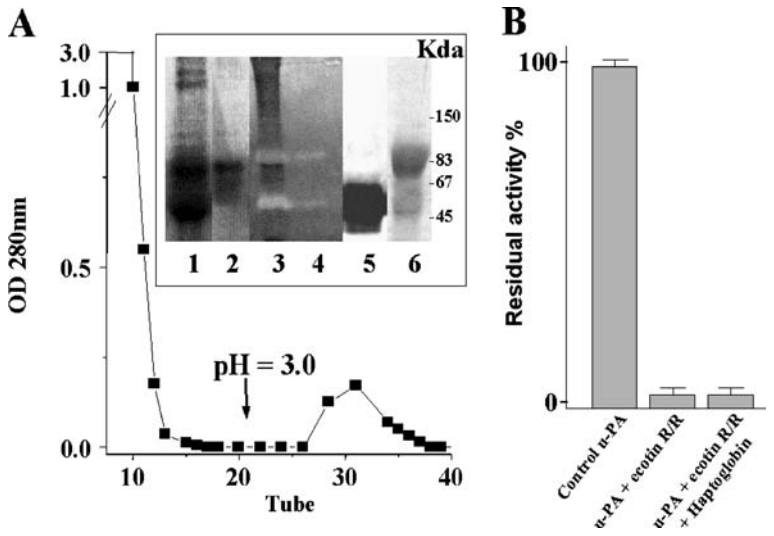

Fig. 3 Analysis of mammary gland lysate using the ecotin-RR affinity column. **A** Elution profile following application of 10 mL of sample to the column. Fractions 2 mL/min. *Arrow* indicates the beginning of the elution at pH 3.0. *Inset* SDS-PAGE (*left*) and substrate gels (*right*). *Lanes 1 and 3* Whole mammary gland; *lanes 2 and 4* lysate material eluted at pH 3.0 from the ecotin-RR column; *lanes 5 and 6* Western blot analysis of retained material on affinity column using u-PA antibodies and trypsin antibodies, respectively. Each lane contains 10 μg of protein. **B** Influence of haptoglobin (2 μM) on the inhibitory effect of ecotin-RR (2 nM) upon hydrolysis of chromogenic substrate by u-PA. Experimental conditions are described in "Materials and Methods." Residual activity is expressed as a percentage of the control ΔA405/min observed in control u-PA. Values are means ± SD of three independent experiments

the zymogen and forming a salt bridge with the N-terminal charged amino group [27]. Our results with ecotin-RR affinity chromatography and immunoassays using trypsin antibodies (3A also inset) reinforced the molecular modeling prediction of others that haptoglobin exhibits a trypsin-like structure [28, 29].

In this work, we tested the effect of human haptoglobin on ecotin inhibition of u-PA (Fig. 3b). Apparently, haptoglobin (2 μM) does not interfere with ecotin-RR binding (2 nM) to u-PA (Fig. 3b). This result suggested that ecotin is interacting with Hp through its secondary binding site, allowing the access of the primary binding site for inhibiting proteases such as u-PA or that the haptoglobin is displaced by uPA. In addition, since (a) in vivo experiments showed that ecotin-RR has a half-life of about 12 h (Selvarajan, personal communication), which is unusually long for peptides and small proteins and (b) haptoglobin does not affect ecotin ability to inhibit proteases, our data also suggest that plasma haptoglobin may serve as a reservoir or carrier for ecotin in blood. Experiments incubating bovine serum with the ecotin-RR column confirmed haptoglobin as the major ecotin binding protein in the plasma, which reinforced this hypothesis (data not shown).

Finally, another source of trypsin-fold serine proteases is the Brazilian snake venom where the enzymes are involved in altering the victim's blood coagulation [17, 33, 34]. Therefore, to identify a thrombin-like enzyme on *L. muta* venom, a snake from the southeast of Brazil, a variant of ecotin was used, namely TSRR/R (V81T, T83S, M84R, M85R, and D70R) that is able to inhibit human α-thrombin.

L. muta venom (50 mg) was first partially purified on a Mono-Q (H10/10) column (Pharmacia). The non-retained material showing protease activity on gelatin gels was

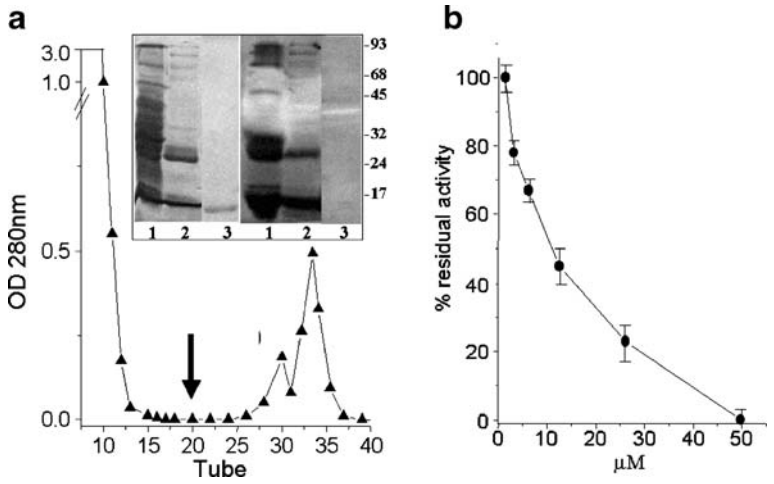

Fig. 4 Analysis of snake venom using an ecotin-TSRR/R affinity column. **A** Elution profile of a partially purified *L. muta venom* the snake venom was partially purified on a Mono-Q column (FPLC) and the non-retained material was applied to an ecotin-TSRR/R affinity column as described in "Materials and Methods." *Arrow* indicates the beginning of the elution step. Inset: SDS-PAGE (*left*) and substrate gel (*right*). *Lane 1* crude venom; *Lane 2* non-retained material from Mono-Q column; *Lane 3* material eluted at pH 3.0 from ecotin-TSRR/R column. Each lane contains 10 μg of protein. **B** Inhibitory effect of ecotin-TSRR/R upon fibrinoclotting activity of purified LM-TL. The enzyme (60 nM) was preincubated for 5 min at room temperature with different concentrations of ecotin-TSRR/R (1–50 μM). Fibrinogen (4 mg/mL) was then added to the assay, and the absorbance at 405 nm was recorded continuously. Residual activity is expressed as a percentage of the control ΔA405/min observed in the absence of the ecotin. Values are means ± SD of three independent experiments

applied to the ecotin-TSRR/R affinity column (Fig. 4). The material eluted at pH 3.0 and exhibited a 16-kDa band on SDS-PAGE (Fig. 4, inset) and a 40-kDa band with catalytic activity on substrate gels (Fig. 4b). The N-terminal sequence of the 16-kDa band (Table 1) revealed 90% identity with the N-terminal sequence of LM-TL, a thrombin-like protein (228 aa) purified from this venom [35]. Since the literature describes the catalytic activity of the proteases of the snake venom proteins that generate peptides or small proteins as a 40-kDa band [36, 37], the inactive 16-kDa band may be a degradation product. In order to verify the identity of the 40-kDa band, we purified LM-TL from *L. muta* venom using the original method [35] and tested for inhibition of its proteolytic activity by the ecotin variant, using a fibrinoclotting assay. Ecotin TSRR/R inhibited LM-TL fibrinoclotting activity with an IC_{50} of 10 μM (Fig. 4c). Since ecotin can form a stable complex with LM-TL, the structural characterization of this enzyme may be facilitated, since ecotin-protease complexes are often easier to crystallize than the proteases alone [29].

Final Remarks

In summary, this work showed that ecotin variants are able to interact with a diverse array of serine proteases and may be used to isolate and characterize proteases from diverse biological samples. This technique should not only assist in the isolation of proteases but may also be useful for identifying proteases and homologous proteins in structural, pharmacological, and biological studies using different biological samples.

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