PROTEASE SECRETED BY THE INFECTIVE LARVAE OF ANGIOSTRONGYLUS CANTONENSIS AND ITS ROLE IN THE PENETRATION OF MOUSE INTESTINE

JUNE-DER LEE AND CHUAN-MIN YEN
Department of Parasitology, Kaohsiung Medical University, Taiwan

Abstract. The infective third-stage larvae of Angiostrongylus cantonensis secrete a proteolytic enzyme that is thought to be essential for both larval penetration into the intestinal wall of the host and full development. Protease activity in these larvae during culture in vitro was determined by zymography, pH optimum, and substrate and inhibitor specificity. Excretory-secretory (ES) products of the third-stage larvae showed protease activity as three bands with molecular masses of 66, 30, and 23 kD by gelatin zymography. The optimal pH value for this protease activity was 10.0. The protease was found to have collagenolytic as well as elastinolytic activity, but these activities were inhibited by serine protease or metalloprotease inhibitors. The importance of this protease in larval penetration of the intestinal wall and entering the blood stream was observed in vitro by cocultured third-stage larvae of A. cantonensis with specific protease inhibitors in the intestines of BALB/c mice. The penetration rates of larvae significantly decreased when serine protease or metalloprotease inhibitors were added to the intestines. These results showed that serine protease and metalloprotease in ES products of A. cantonensis third-stage larvae are associated with larval penetration of the intestinal walls of mice.

INTRODUCTION

During the life cycle of Angiostrongylus cantonensis, the infective third-stage larva (L3) are ingested into the lumen of non-permissive hosts, including humans, migrate via the venous system into the lungs, and then develop into the fifth-stage larvae in the brain, inducing diseases such as eosinophilic meningencephalitis.1 The parasites must overcome the immune mechanisms of their host, and migrate to their final destination, where they can mature. It is very important for these larvae while they are in the intestine to penetrate the intestinal wall and enter the venules. Parasite proteases, some of which are found in excretory-secretory (ES) products, facilitate the invasion of host tissues,2 aid in the digestion of host proteins,3 help parasites evade the host immune response,4 and mediate molting in parasitic nematodes.5 Infective larvae of A. cantonensis penetrate the mucosa of the small intestines and pass via the circulating system to the brain, where they molt. Since the larvae do not possess a buccal stylet, mucosal penetration is probably facilitated by tissue-degrading proteases.

Protease secreted by infective larvae, which facilitates penetration of the skin or the intestinal wall of humans, has been observed in some helminths.6 In this study, we determined when a non-permissive host is infected with A. cantonensis whether secretion of protease is an important factor in infective larval penetration of the intestinal wall, entry into the blood stream, and migration into the brain of the host. Third-stage larvae were collected from snails after artificial digestion and cultivated in vitro to obtain ES products. Protease in ES products was identified by gelatin zymography. The optimum pH for protease activity was determined by using a universal substrate in buffered solutions of different pHs. Proteolytic activity was determined by using peptides of low molecular masses and protease inhibitors. The effect of protease inhibitors on intestinal wall penetration by L3 of A. cantonensis was investigated in an in vitro study.

MATERIALS AND METHODS

Parasite. The Taiwan strain of A. cantonensis used in this study was originally isolated from the giant African snail (Achatina fulica) collected in Ping-tung County in southern Taiwan. Angiostrongylus cantonensis is maintained in our laboratory by cycling through the planorbid snail ( Biomphalaria glabrata) and Sprague-Dawley rats. First-stage larvae of A. cantonensis were recovered from infected rat feces and fed to snails.7 The L3 of A. cantonensis were obtained from the tissues of infected snails that had been artificially digested with 0.6% pepsin-HCl (pH 2–3) for one hour.8 Rats were then infected with L3 by stomach intubation.

Excretory-secretory medium. Third-stage larvae were surface sterilized by incubation in 0.25% NaOCl for five minutes at room temperature. They were then washed six times in 0.15 M phosphate-buffered saline, pH 7.2, containing gentamicin (72 µg/mL). The larvae were incubated at a concentration of 10,000 larvae/mL in RPMI 1640 medium supplemented with gentamicin (72 µg/mL) and 2 mM L-glutamine at 27°C in an atmosphere of 5% CO2. The medium was changed every 24 hours by centrifuging the L3 at 800 × g for three minutes and resuspending them in fresh complete medium. Motility of the L3 was checked with an inverted microscope, and the culture was terminated when mobility decreased below 95%. The collected medium was passed through a 0.22-µm filter (Milipore, Eschborn, Germany), then concentrated 50–100 fold using 10-kD exclusion size membranes (Centricon; Amicon Corp., Danvers, MA). The resulting aliquots were then stored at −70°C.

Zymography. Substrate gel electrophoresis was carried out as described previously.9 A 10% polyacrylamide running gel polymerized in the presence of 0.1% gelatin (swine skin type A; Sigma, Deisenhofen, Germany) and 4% stacking gel without gelatin were used. Cultured medium, collected as mentioned above, was mixed with an equal volume of sample buffer (5% sodium dodecyl sulfate [SDS], 2% sucrose, 0.005% bromphenol blue in 0.5 M Tris-HCl, pH 6.8, containing 0.4% SDS stacking gel buffer). Unboiled samples were subjected to electrophoresis in Tris-glycine buffer, pH 8.3, at 12.5 mA per gel. Following electrophoresis, the SDS was removed by incubation with 2.5% Triton X-100 for two hours at 25°C, and the gel was then incubated in neutral buffer (0.1 M Tris-HCl, pH 7.8 containing 1 mM CaCl2) at 37°C for 24 hours. The gel was stained with Coomassie blue, and protease
bands were identified as clear areas in the gel after being destained with destaining solution (40% methanol, 10% acetic acid).

**Effect of pH on protease activity.** Proteolytic activity was measured spectrophotometrically by use of chromogenic substrates and universal protease substrate (azocasein; Roche, Mannheim, Germany) after incubation at 37°C in buffer with a pH range of 4–11. Reaction mixtures were routinely composed of 100 µL of ES medium, 50 µL of incubation buffer (0.2 M Tris-HCl, 0.02 M CaCl2), and 50 µL of substrate (4 mg/mL). Samples were incubated for 16 hours and the reaction was stopped by adding 480 µL of Stop reagent (5% trichloroacetic acid). After an additional incubation at 37°C for 10 minutes, the solution was centrifuged at 10,000 × g for five minutes. Four hundred microliters of supernatant was then mixed with 600 µL of assay buffer (0.5 M Tris-HCl, pH 8.8). Absorbance of the sample was immediately measured at 574 nm, using a spectrophotometer (DU 7400; Beckman, Palo Alto, CA) at room temperature. All assays were done in triplicate.

**Determination of protease type.** The types of protease activity measured in this study and their relative low molecular mass substrates are summarized in Table 1. All low molecular mass substrates (Sigma) were prepared in dimethylsulfoxide (DMSO) at a concentration of 1 mg/mL. Reaction mixtures were routinely composed of 25 µL of ES medium, 100 µL of 100 mM phosphate buffer, pH 6.8, 50 µL DMSO, and 5 µL of substrate solution. The activities of proteases in low molecular mass substrates were measured at 405 nm after incubation for one hour. All activities were corrected for nonenzymic hydrolysis by subtraction of their reagent blanks. All assays were done in triplicate.

**Effects of inhibitors on protease activity.** Collagenolytic activity was measured spectrophotometrically using the chromogenic substrate Azocoll (Sigma) after incubation in 100 mM Tris-HCl, pH 7.8, for 18 hours at 37°C. Reaction mixtures were routinely composed of 50 µL of ES medium, 100 µL of buffer containing gentamicin (5 mg/mL), and 50 µL of substrate (5 mg/mL). Absorbance of the reaction mixtures was measured at 520 nm and protease activity was calculated by subtraction of reagent blanks containing medium alone. Elastinolytic activity was measured spectrophotometrically by cleavage of insoluble bovine elastin (Sigma). Fifty microliters of ES medium were then incubated overnight at 37°C with 20 mg/mL of elastin (20 mg/mL) in 100 mM Tris-HCl, pH 7.8. Released peptides were analyzed determined by the ninhydrin method and optical density was read at 570 nm.10 Various protease inhibitors were tested for their effects on protease activities. The inhibitors were used at the following concentrations: 100 µM aprotinin (Sigma), 10 mM ethyl-enediaminetetraacetic acid (EDTA; Sigma), 1 mM L-trans-epoxyysuccinyl-leucylamide-(4-guanidino)-butane (E64; Sigma), and 100 µg/mL of pepstatin (Sigma). For substrate degradation, the ES medium was incubated with inhibitors for 30 minutes at the final concentration cited above before the respective substrate was added. All assays were done in triplicate. The effect of inhibitors was expressed as

\[
\% \text{ Residual activity} = \frac{\text{Inhibited mean absorbance}}{\text{Uninhibited mean absorbance}} \times 100
\]

**Affect of protease inhibitors on the intestinal penetration of L3.** Fresh duodenum was excised from three-week-old BALB/c mice and the mesenteric venous plexuses were carefully preserved. They were washed with normal saline to clean the lumen and immediately placed in a petri dish containing RPMI 1640 medium plus 10% fetal calf serum at 37°C. One hundred L3 were incubated in 1 mL of single specific inhibitor solution or combined with protease inhibitor solution for 30 minutes, then introduced into the excised intestine via the openings. The two open ends were then tied with string. The control group was mouse intestine containing 100 L3 in 1 mL of RPMI 1640 medium alone. The apparatus was placed in an incubator with a gas phase of 5% CO₂ and 95% air for four hours at 37°C. The L3 remaining in the lumen were washed and counted at the end of the incubation period. The working concentration of inhibitor solutions in this assay was 10 mM aprotinin, 1 M EDTA, 100 mM E64, and 10 mg/mL of pepstatin. The effect of inhibitors was expressed as

\[
\% \text{ Inhibition rate} = \frac{\text{No. of L3 in the lumen with inhibitor solution}}{100 \text{ No. of L3 in the lumen without inhibitor}} \times 100
\]

**RESULTS**

**Proteases in ES products of L3.** The ES medium from L3 larvae was screened for protease activity by gelatin zymography. The protease activity dissolves the gelatin of the running gel and appears as clear bands. Analysis of ES products of L3 in gelatin–polyacrylamide gel electrophoresis clearly showed three clear bands of protease activity with molecular masses of 66, 30, and 23 kD (Figure 1). No proteolytic band was detected from medium without L3.

**Effect of pH on protease activity.** Protease activity in ES products from *A. cantonensis* L3 was detected by colorimetric assays using a universal protease substrate. The peak of protease activity was over a range of pH 4.0–11.0 (Figure 2). The maximum activity occurred at pH 10.0, with an activity ≥ 300 milli-absorbance units within a range of 7.0–10.0, demonstrating the stability of protease activity in basic conditions.

**Determination of protease type.** The proteolytic activity of ES products of the infective larvae of *A. cantonensis* was analyzed to determine the types of proteases digesting proteins, as well as a series of low molecular mass substrates, as detailed in Table 1. The results indicated that proteolytic activity of ES products reacted highly to collagenolytic
(azocoll), and elastinolytic (Cbz-L-Ala-4-NPE, Cbz-β-Ala-4-NPE) substrates, but only slightly to tryp tic (Cbz-L-Lys-4-NPE, Cbz-L-Arg-4-NPE), and chymotryptic (Cbz-L-Tyr-4-NPE, Cbz-L-Trp-4-NPE, Cbz-L-Phe-4-NPE, Bz-L-Tyr-4-NA) substrates. The proteases in ES products of A. cantonensis L3 are responsible for collagen-type and elastase-type activities.

**Effects of protease inhibitors on the azocollytic and elastinolytic activities.** Table 2 shows the results when protease inhibitors are used to determine the class of proteases in A. cantonensis L3 ES products by using azocoll and elastin as substrates. With azocoll as the substrate, proteolytic activity in ES products of A. cantonensis L3 was markedly inhibited by aprotinin (residual activity = 9%), an inhibitor of serine protease, and slightly inhibited by the metalloprotease inhibitor EDTA (residual activity = 28%). In contrast, the cysteine protease inhibitor E64 and the aspartic protease inhibitor pepstatin had only mild effects on ES products (residual activities = 65% and 91%, respectively). The proteolytic activity in ES products was also markedly inhibited by aprotinin (residual activity = 7%), an inhibitor of serine protease, and slightly inhibited by the metalloprotease inhibitor EDTA (residual activity = 31%) when using elastin as substrate. In contrast, the aspartic protease inhibitor pepstatin and the cysteine protease inhibitor E64 had only mild effects on ES products (residual activities = 81% and 89%, respectively). This indicates that the class of proteases in ES products of A. cantonensis are serine protease and metalloprotease.

**Affect of protease inhibitors on the intestinal penetration of L3.** If the proteases secreted by L3 are essential for enabling larvae to penetrate the intestinal wall, then inhibition of this enzyme should arrest larval invasion. Larval invasion of intestine was observed in the presence of one, two, three, or all protease inhibitors (Table 3). Of the single specific protease inhibitor of larvae penetration assayed, serine protease inhibitor had the highest inhibition rate (51%). Metalloprotease and aspartic protease inhibitors had inhibition rates of 20% and 13%, respectively, whereas cysteine protease inhibitor had no effect on inhibition of larvae penetration. Of two combined protease inhibitors assayed for the inhibition of larvae penetration, serine protease inhibitor combined with aspartic protease inhibitor had the highest inhibition rate (61%), followed by serine protease inhibitor combined with metalloprotease inhibitor (59%) and serine protease inhibitor combined with cysteine protease inhibitor (46%). Metalloprotease inhibitor combined with aspartic protease inhibitor and cysteine protease inhibitor combined with

**TABLE 2**

<table>
<thead>
<tr>
<th>Collagenolytic activity</th>
<th>Optical density (at 520 nm)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3 ES (50 μL)</td>
<td>270</td>
<td>100</td>
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<tr>
<td>L3 ES (50 μL) aprotinin (100 μM)</td>
<td>25</td>
<td>9</td>
</tr>
<tr>
<td>L3 ES (50 μL) E-64 (1 mM)</td>
<td>176</td>
<td>65</td>
</tr>
<tr>
<td>L3 ES (50 μL) EDTA (10 mM)</td>
<td>75</td>
<td>28</td>
</tr>
<tr>
<td>L3 ES (50 μL) pepstatin (100 μg/ml)</td>
<td>977</td>
<td>91</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Elastinolytic activity</th>
<th>Optical density (at 570 nm)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3 ES (50 μL)</td>
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<td>100</td>
</tr>
<tr>
<td>L3 ES (50 μL) aprotinin (100 μM)</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>L3 ES (50 μL) E-64 (1 mM)</td>
<td>278</td>
<td>89</td>
</tr>
<tr>
<td>L3 ES (50 μL) EDTA (10 mM)</td>
<td>96</td>
<td>31</td>
</tr>
<tr>
<td>L3 ES (50 μL) pepstatin (100 μg/ml)</td>
<td>252</td>
<td>81</td>
</tr>
</tbody>
</table>

* All assays were done in triplicate.
† % Residual activity = inhibited mean absorbance/uninhibited mean absorbance x 100.
aspartic protease inhibitors had inhibition rates of 24% and 16%, respectively, while cysteine protease inhibitor combined with metalloprotease inhibitors had the lowest inhibition rate (15%). Of the combinations of three protease inhibitors, mixtures of serine, cysteine, and metalloprotease inhibitors had the highest inhibition rate of larvae penetration (56%), followed by serine, aspartic, and metalloprotease inhibitors (55%) and serine, cysteine, and cysteine protease inhibitors (43%), respectively. The mixtures of cysteine, aspartic, and metalloprotease inhibitors had the lowest inhibition rate (29%). When all protease inhibitors were combined, the inhibition rate was 69%.

DISCUSSION

Three bands were observed in gelatin zymography after proteolysis by ES products from *A. cantonensis*. This means that proteases secreted by *A. cantonensis* infective larvae exhibit diversity in the classes of proteases, based on the differential migration in polyacrylamide gels containing gelatin after sodium dodecyl-sulfate-polyacrylamide gel electrophoresis. Multiple proteases in ES products have also been reported in other parasites, including *Toxocara canis*, *Ascaris suum*, and *Fasciola hepatica*. The heterogeneity of ES proteases may reflect functional variability and/or conditions under which these proteases are active. Homogenized adult worms of *A. cantonensis* showed two kinds of acidic protease or metalloprotease in the degradation of extracellular matrix components. The optimum pH for proteases activity of infective larvae from *A. cantonensis* infective larvae reached its highest level in buffered solution with a pH of 10.0. The optimum alkaline pH of the protease activity is compatible with the known pH of portions of the intestinal mucosa, such as those in the duodenum associated with Brunner’s gland secretions (pH 8.9–9.3). Proteases from *A. cantonensis* may be activated due to the change of pH when infective larvae enter the duodenum through the stomach.

Proteases are most readily categorized into four groups using specific inhibitors for detecting each of the four protease classes. This study has extensively discussed the use of inhibitors in helping to determine the biologic functions of proteases. Elastolytic activity has been confirmed in ES products of *A. cantonensis* infective larvae, in view of the ability of these products to degrade elastin *in vitro*. Elastase in ES products may be involved in degrading components of the intestinal extracellular matrix proteins of host tissues. However, elastin is also a major macromolecular component of the tissue barriers (e.g., dermal extracellular matrix), and is rarely found in intestinal tissue. It is also known that intestine wall invasion by *Anisakis simplex* and *Entamoeba histolytica* is due to the secretion of histolytic proteases, not elastases. Moreover, serine and metalloproteases were found in ES products of *A. cantonensis* infective larvae in the collagenolytic substrate inhibition assay, and involvement of serine protease or metalloprotease in the degradation of extracellular matrix components has been reported in other helminths. Metalloproteases (e.g., elastases and collagenases) are a diverse group of enzymes that use a metal ion, usually zinc, in catalysis, and have a powerful degrading effect on extracellular matrix components.

When parasites remain in the host, they may use proteases for functions other than digestion, such as host penetration, tissue migration, immune evasion, and molting. Indeed, more than 65% of *A. cantonensis* L3 lost their ability to penetrate the intestinal wall when four types of protease inhibitors were mixed with a larval suspension and incubated in a separate mouse intestines *in vitro*. Nevertheless, 31% of *A. cantonensis* L3 still penetrated the intestinal wall when all of the protease inhibitors were present. Thus, *A. cantonensis* L3 may pen-

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Class of protease inhibited</th>
<th>L3 no.</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Serine</td>
<td>5</td>
<td>51</td>
</tr>
<tr>
<td>Aprotinin, E64</td>
<td>Cysteine</td>
<td>48</td>
<td>51</td>
</tr>
<tr>
<td>EDTA</td>
<td>Metallo</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Peptatin, E64</td>
<td>Aspartic</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Aprotinin, EDTA</td>
<td>Serine, cysteine</td>
<td>44</td>
<td>46</td>
</tr>
<tr>
<td>Aprotinin, Peptatin, E64</td>
<td>Serine, metallo</td>
<td>56</td>
<td>59</td>
</tr>
<tr>
<td>E64, EDTA</td>
<td>Cysteine, metallo</td>
<td>58</td>
<td>61</td>
</tr>
<tr>
<td>E64, Peptatin</td>
<td>Cysteine, aspartic</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>EDTA, Peptatin, E64</td>
<td>Metallo, aspartic</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Aprotinin, E64, EDTA</td>
<td>Serine, cysteine, metallo</td>
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<td>56</td>
</tr>
<tr>
<td>Aprotinin, E64, Peptatin, E64</td>
<td>Serine, cysteine, aspartic</td>
<td>41</td>
<td>43</td>
</tr>
<tr>
<td>Aprotinin, EDTA, Peptatin</td>
<td>Serine, metallo, aspartic</td>
<td>52</td>
<td>55</td>
</tr>
<tr>
<td>E64, EDTA, Peptatin, E64</td>
<td>Cysteine, metallo, aspartic</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>Aprotinin, EDTA, Peptatin</td>
<td>Serine, cysteine, metallo</td>
<td>66</td>
<td>69</td>
</tr>
</tbody>
</table>

* Inhibitor concentrations were as follows: 10 mM aprotinin, 100 mM L-trans-epoxysuccinyl-leucylamide-(4-guanadino)-butane (E64), 1 M ethyl-enediaminetetraacetic acid (EDTA), and 10 mg/mL of pepstatin. Six mice in each group were tested.

† Inhibition rate (%) = no. of L3 in the lumen with inhibitor solution/(100 – no. of L3 in the lumen without inhibitor) × 100.
etrate the intestinal wall by both secretion of proteases and the mechanical movement of the larvae. In fact, when ingested by humans, *Anisakis simplex* uses a boring tooth to drill holes in the stomach and intestinal wall, together with the secretion of proteases to degrade tissue components. However, since there is no buccal stylet on *A. cantonensis* L3 to assist in the penetration of the intestinal wall, secretion of proteases is more important than mechanical movement when these parasites invade hosts. In this study, we found that the penetrating intestine wall activity of proteases from *A. cantonensis* infective larvae was prominently inhibited by serine protease inhibitor, regardless of whether single or multiple protease inhibitors were used. We therefore concluded that serine protease secreted from *A. cantonensis* infective larvae participates in the infection route of intestinal penetration in the host.

Araki and others developed an *in vitro* model to evaluate the penetration of the intestinal wall of *A. cantonensis* L3 by using a piece of open rat duodenum to cover a the crosscut surface of a disposable syringe. A five-hour incubation was required to obtain a penetration rate of approximately 85%. In this study, we excised entire duodenums of BALB/c mice into which L3 had been injected. Only four hours of incubation were required for a penetration rate of 95%. This was a time-saving and easy method of evaluating the activity of both proteases from *A. cantonensis* L3 and their inhibitors. It was noted that the highest inhibition rate was only 51% when single specific proteases inhibitors were used to inhibit the intestine penetrating activity of *A. cantonensis* L3 in this *in vitro* model. The concentration of protease inhibitor may not have been sufficient to completely block the continuously secreted proteases from incubating larvae. If the concentrations of protease inhibitors were elevated, penetration of larvae through the intestine *in vitro* might be effectively inhibited, but the test would be unreliable because the biologic activity of the intestine might also be simultaneously damaged.

Some studies reported that parasite secret various enzymes, including lipases, a fibronectin-like enzyme, and superoxide dismutase, in the host. Furthermore, protease secretion has been detected in rats and humans infected with *Strongyloides* sp. and dogs infected with *Ancylostoma caninum*. Our results show that the proteases secreted by *A. cantonensis* L3 share characteristics of alkaline serum/metalloproteases and attributes of serine protease to facilitate the duodenal penetration of the host. This conclusion is supported by 1) distribution of secreted proteases by zymography, 2) maximum activity at alkaline pH, consistent with *in situ* conditions of the duodenum, 3) pronounced substrate inhibition of activity by serine or metalloprotease inhibitors, and 4) inhibition of intestinal penetration activity by serine protease inhibitor.

Received June 28, 2004. Accepted for publication December 16, 2004.

Acknowledgments: We thank L. Y. Chuang and S. G. Yang for their help in assaying the *in vitro* penetration of excised intestinal wall of *A. cantonensis* infective larvae.

Financial support: This study was supported by grant no. NSC-85-2331-B-037-015 from the National Science Council, Taiwan, Republic of China.

Authors’ address: June-Der Lee and Chuan-Min Yen, Department of Parasitology, Kaohsiung Medical University, No. 100 Shih-Chuan 1st Road, Kaohsiung City, Taiwan, Republic of China, Telephone: 886-7-312-1101-2169, Fax: 886-7-321-8309, E-mail: chmiye@kmu.edu.tw

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